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The effects of glycerol ingestion on body water distribution and exercise performance

by

George Aphamis

A Doctoral Thesis

**Submitted in Partial Fulfillment of the Requirements for Award of
Doctorate of Philosophy at Loughborough University**

November 2011

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List of Abbreviations

ANOVA	analysis of variance
bpm	beats per minute
Cl ⁻	chloride
CV	coefficient of variation
°C	degrees Celsius
g	grams
h	hour
H ⁺	hydrogen
K ⁺	potassium
K ₂ EDTA	potassium ethylenediamine tetra acetic acid
kg	kilogram
l	litre
m	metre
M	mole
mg	milligram
min	minute
ml	millilitre
mmol	millimole
mosm	milliosmole
MVC	maximum voluntary contraction
PCr	phosphocreatine
Pi	phosphate
Na ⁺	sodium
NaCl	sodium chloride
RM	repetition maximum
s	second
SD	standard deviation
VO _{2max}	maximum oxygen uptake
W	watts
y	years

Abstract

Water movement in the body is determined by the osmotic forces acting on the cell membrane. Ingestion of a highly-hypertonic glycerol solution resulting in high extracellular osmolality could drive water out of the intracellular space into the vascular space resulting in reduced muscle hydration and increased blood volume. The aim of this thesis was to study the effects of altered body water distribution during exercise. In chapter 3, ingestion of a 400 ml glycerol solution ($1 \text{ g}\cdot\text{kg}^{-1}$ body mass) increased serum osmolality ($309 \text{ mosmol}\cdot\text{kg}^{-1}$) which was associated with a 4.0% increase in blood volume due to a 7.2% increase in plasma volume, attributed to a shift of water from the intracellular space, resulting presumably in tissue dehydration. Glycerol ingestion was then used as a means of altering body water distribution in the other studies described in this thesis.

Altered body water distribution had no acute effect on force production during quadriceps muscle isometric exercise (chapter 4), or handgrip strength (chapter 5). Regarding chronic effects (chapter 5), two groups of participants exercised handgrip and initiated recovery after ingestion of either a glycerol solution or placebo over a period of 8 weeks. Maximum handgrip strength increased in both groups and there was no statistically significant difference between the two groups.

In chapters 6 and 7, the subjects performed a cycling exercise protocol to fatigue. In the glycerol trial, time to fatigue decreased compared with the iso-osmotic trial during an incremental VO_2max test (chapter 6) and during cycling against a constant load at 100% VO_2max intensity (chapter 7). In the glycerol trial, there was an accelerated increase in blood lactate and an accelerated increase in serum potassium (chapter 7), indicating altered muscle metabolism which may have contributed to the early development of fatigue.

Keywords: glycerol, body water, muscle hydration, isometric exercise, strength, handgrip, strength training, VO_2max , time to fatigue, cycling

Chapter 1

General Introduction

Body water

Total body water averages about 50 – 60% of body mass. This equates to approximately 35 – 42 l for an average 70 kg male. Total body fluid is distributed among two major compartments: the extracellular fluid and the intracellular fluid. The intracellular fluid (which is inside the body cells) amounts to about 40% of total body mass and the extracellular space (fluids outside the cells) accounts for about 20% of body mass (Guyton & Hall, 1996).

Extracellular space is then divided into two compartments: the interstitial space, which makes up about three-fourths of the extracellular fluid (15% total body mass) and the plasma, which makes up almost one-fourth of the extracellular space (5% total body mass) (Guyton & Hall, 1996). Plasma is the non-cellular part of the blood and communicates continuously with the interstitial fluid through the pores of the capillary membranes. The relative amounts of extracellular fluid distributed between the plasma and interstitial spaces are determined mainly by the balance of hydrostatic and osmotic forces. The distribution of water between the intracellular and extracellular space is determined mainly by the osmotic effect of the solutes acting across the cell membrane. These solutes can be inorganic ions such as Na^+ , K^+ , or Cl^- , or organic molecules such as amino acids, taurine, alanine, proline, glycerol, sorbitol, or myo – inositol (Strange, 2004). Changes in intracellular or extracellular solute content generate a transmembrane osmotic gradient and water moves across the cell membrane rapidly so that the intracellular fluid remains isotonic with the extracellular fluid (Guyton & Hall, 1996). This water movement may alter cellular hydration by causing cell swelling or shrinkage.

In a series of experiments in the 1990's, Haussinger and his group (Berneis et al., 1999; Haussinger et al., 1991; Hallbrucker et al., 1991a; Hallbrucker et al., 1991b; Haussinger et al., 1990a,b; Haussinger et al., 1994; Stoll et al., 1992; Low et al., 1996; vom Dahl & Haussinger, 1996; vom Dahl et al., 1991) studied cell metabolism under different states of increased and decreased hydration.

Exposing cells to a hypo-osmotic environment led to an influx of water into the cell,

resulting in cell swelling, and exposing cells to a hypertonic environment led to cell shrinkage. They observed that cell volume increase favoured anabolic processes such as protein and glycogen synthesis, whereas cell shrinkage had the opposite effect (Haussinger et al., 1994).

If this theory applies in a whole body situation it could affect muscle function since water is the organic solvent of most biochemical processes and adequate hydration is important for exercise performance; extracellular and intracellular fluid volumes affect such physiological processes as transport of respiratory gases and energy substrates, as well as energy metabolism (Schoffstall et al., 2001).

Cellular hydration and cell volume theory

Proper cell hydration is important as cells need to maintain a constant volume for normal cell function and survival (Strange, 2004). The cellular hydration state is not maintained in absolute constancy, but it is dynamic and changes within minutes under the influence of aniso-osmolality, hormones, nutrients and oxidative stress (Haussinger et al., 1994; Haussinger, 1996). Factors leading to cell swelling are, for example, extracellular hypo-osmolality, elevated plasma insulin and elevated plasma IGF-1 (Hallbrucker et al., 1991a; Hallbrucker et al., 1991b; Haussinger et al., 1991; vom Dahl & Haussinger, 1996). Elevated glutamine and plasma amino acids also lead to cell swelling due to a cumulative uptake of glutamine and amino acids into the cell, which creates an osmotic gradient and water flux into the cell (Hallbrucker et al., 1991b; Haussinger et al., 1990b). Other factors such as hyper-osmotic plasma, increased plasma glucagon concentrations and oxidative stress induce cell shrinkage (Haussinger, 1996; Keller et al., 2003; Schliess & Haussinger, 2002). In time, the cells regain almost their original size (Haussinger et al., 1994). Following cell swelling, the cells display a regulatory volume decrease and following cell shrinkage, regulatory volume increase mechanisms restore cell volume. Cell volume is regulated by the loss or gain of osmotically active solutes such as Na^+ , K^+ and Cl^- . Regulatory

volume decrease is achieved via the loss of K^+ , Cl^- and HCO_3^- , whereas regulatory volume increase is achieved through Na^+ , K^+ , Cl^- uptake via activation of Na^+-H^+ antiport activity, $Na^+ - K^+ - 2Cl^-$ cotransport and $Cl^- - HCO_3^-$ exchanges (Haussinger et al., 1994; Haussinger, 1996; Keller et al., 2003). Then, activation of the $Na^+ - K^+ - ATPase$ serves to replace accumulated Na^+ with K^+ (Lang et al., 1998).

In vitro, the cell regulatory volume mechanisms do not completely restore the initial cell volume (leaving the cells in either a slightly shrunken or swollen state) and the extent of this volume deviation has been shown to act as a signal modifying cellular behaviour (Haussinger et al., 1994). Cell swelling favours the synthesis and inhibits the degradation of proteins, glycogen and, to a lesser degree, lipids (Lang et al., 1998). Cell shrinkage has the opposite effect (Haussinger et al., 1994; Lang et al., 1998). Overall, cell swelling promotes growth factor-like effects and cell shrinkage supports a catabolic situation (Haussinger et al., 2004).

Although most of the research was conducted on hepatocytes, the cell volume theory as suggested by Haussinger appears to also hold true for skeletal muscle cells and mitochondria (Lang et al., 1998).

In vivo, clinical observations are in agreement with the cell volume theory. Finn et al. (1996) observed that progressive cellular dehydration is associated with loss of protein and potassium from body stores in critically ill patients suffering from major trauma or sepsis. Further, in diabetic individuals, protein catabolism is associated with dehydration paralleled by hyperglycaemia that results in plasma hyper-osmolality, which may lead to cell shrinkage (Hellerstein, 1995; Lang, 2007).

Cell volume and protein metabolism

Increased intracellular water space (increased cell volume) has been shown to stimulate overall protein synthesis (Stoll et al., 1992) and to inhibit proteolysis (Hallbrucker et al., 1991a; Hallbrucker et al., 1991b; Haussinger et al., 1990; vom Dahl et al., 1991).

Haussinger et al. (1990a) exposed perfused rat liver in hypo- and hyper-osmotic conditions. Control osmolality was $305 \text{ mosmol} \cdot \text{kg}^{-1}$, the hypo-osmotic condition was set at $225 \text{ mosmol} \cdot \text{kg}^{-1}$ and hyper-osmolality was at $385 \text{ mosmol} \cdot \text{kg}^{-1}$.

Following exposure to hypo-osmotic conditions, liver mass increased about 20%. Following a K^+ efflux and a regulatory volume decrease, liver mass came to a steady state that was greater than the starting value. As indicators of proteolytic rate, the investigators used the release of branched chain amino acids from the cells (since branched chain amino acids are not synthesized nor catabolised in rat liver). Their results showed that hypo-osmotic exposure of rat liver resulted in a decrease of branched chain amino acid release (~ 22%) which was reversible upon re-exposure to normo-osmotic perfusion media. Exposure of rat liver to hyper-osmolality resulted in an increased (22%) release of branched chain amino acids. These data demonstrated that cell swelling inhibited proteolysis and cell shrinkage promoted protein breakdown.

Haussinger et al. (1990b) reported that cell swelling can also be elicited by increases in intracellular glutamine concentration. Exposure of perfused rat liver to iso-osmotic media containing glutamine led to an increased uptake of glutamine. This resulted in cell swelling, which was accompanied by an inhibition of leucine released from the liver. This demonstrated an inhibition of proteolysis by glutamine. Other amino acids, such as alanine, serine and glycine, have also been shown to elicit liver cell swelling and to be potent inhibitors of proteolysis (Haussinger et al., 1990a; vom Dahl & Haussinger, 1996).

Hallbrucker et al. (1991b) investigated the relationship between proteolysis (as expressed by leucine release) and amino acid induced cell swelling on isolated perfused liver. An infusion of glutamine or glycine individually led to decreased proteolysis, which was strongly dependent upon the increase in liver mass. The infusion of alanine, serine, proline or phenylalanine, or a mixture of these amino acids also led to a decrease in leucine release, suggesting inhibition of proteolysis; however, the effect of these amino acids was only partially explained by changes in cell volume. The antiproteolytic effect of the amino acids glycine and glutamine could be mimicked quantitatively by equipotent hypo-osmotic cell swelling.

Cell swelling is also induced by the action of insulin, which results in K^+ uptake and Haussinger et al. (1991) suggested that the antiproteolytic action of insulin is dependent on the extent of the simultaneously occurring changes of the

intracellular water space in perfused rat liver. Further research by Hallbrucker et al. (1991a and 1991b) and vom Dahl and Haussinger (1996) demonstrated that insulin and IGF –1 do result in K^+ accumulation in the cells that leads to increases in cell volume, showing that the antiproteolytic effect of insulin and IGF – 1 reflects mainly their cell swelling potency.

Cell volume and carbohydrate metabolism

Cell swelling leads to decreased lactate and pyruvate release from the cell, whereas exposure of cells to hyper-osmotic perfusates results in an augmented release of lactate and pyruvate (Lang et al., 1989). This was an early finding that changes in extracellular osmolality affect cell carbohydrate metabolism. Low et al. (1996) investigated whether muscle glycogen synthesis was modulated by changes in cell volume by studying myotubes in primary culture exposed to sucrose solutions of different osmolality with or without insulin. Their results showed that in the absence of insulin, skeletal muscle glycogen synthesis in the hypo-osmotic condition ($170 \text{ mosmol}\cdot\text{kg}^{-1}$) increased by 75%, whereas it decreased by 31% in the hyper-osmotic condition ($430 \text{ mosmol}\cdot\text{kg}^{-1}$) compared with the iso-osmotic condition ($300 \text{ mosmol}\cdot\text{kg}^{-1}$). The addition of insulin to the iso-osmotic media also increased glycogen synthesis by 53% compared with the control value. Further, hypo-osmotic exposure resulted in increased glycogen synthesis by 40% in cells already stimulated by supramaximal insulin concentrations. This showed that the anabolic effects of insulin and cell swelling were at least partly additive.

Glycogen synthesis is controlled by the activity of glycogen synthase and acetyl – CoA carboxylase. Baquet et al. (1991) investigated the action of these enzymes in parallel with the increased glycogen synthesis observed after cell swelling in hepatocytes. Their results showed that exposure to hypo-osmotic perfusates increased cell volume and led to an activation of glycogen synthase and acetyl-CoA carboxylase, whereas exposure to hyper-osmotic media inhibited the action of both enzymes.

Exposure of skeletal muscles to hyper-osmotic conditions

Exposure of muscle fibres to hypertonic solutions is known to lead to cell dehydration and shrinkage (Blinks, 1965; Bozler, 1965). Caputo (1968) exposed single muscle fibres in hypertonic solutions containing glycerol ($460 \text{ mosmol}\cdot\text{kg}^{-1}$) and investigated changes in volume and muscle twitch. The fibres shrank rapidly following exposure to the hypertonic solution and twitch tension fell by 25%. These effects were transient and were reversed on return to the isotonic medium. Even though the author could not clearly demonstrate what the underlying mechanism was, he suggested that the fibre volume changes that occur in hypertonic solutions appeared to be the cause of the alterations in twitch tension up to a certain extent. However, he did point out that cellular dehydration would cause changes in ionic strength that could also affect the contractile mechanism of the cell. A decline in twitch tension was also observed by Gordon and Godt (1970) following exposure of frog skeletal muscles to hypertonic conditions, which was attributed either to the increases in ionic strength following dehydration or a direct effect of cellular dehydration on the contractile proteins. This could be attributed either to a failure in the process linking the depolarisation of the muscle membrane to calcium release from the sarcoplasmic reticulum or the inability of actin and myosin to move normally in the dehydrated cell (Miyamoto & Hubbard, 1972). Later evidence by Rapoport et al. (1982) showed that exposure of muscle fibres to a hyper-osmotic environment ($570 \text{ mosmol}\cdot\text{kg}^{-1}$) inhibited contractility of the fibres when these were electrically stimulated. The authors suggested that this could be due to decreased release and re – uptake of calcium during electrical stimulation. Furthermore, Rapoport et al. (1982) also reported reduced phosphocreatine and ATP concentration, and increased glucose – 6 – phosphate concentration, suggesting increased glycogenolysis, during immersion of the fibres in the hyper-osmotic media in resting conditions. These findings showed that hypertonicity affected the resting metabolism of the muscle fibres. Recent evidence by Antolic et al. (2007) investigated the effects of different osmotic conditions on intact resting skeletal muscles. The muscles were incubated in either iso – osmotic (control, $290 \text{ mosmol}\cdot\text{kg}^{-1}$), hypo–osmotic (190

mosmol·kg⁻¹) or hyper-osmotic (400 mosmol·kg⁻¹) media. The results showed a 3% increase in muscle water in the hypo-osmotic condition and a 3% loss of water in the hyper-osmotic condition. This was accompanied by an increase in cross sectional area in the hypo-osmotic condition and a reduction in cross sectional area in the hypertonic condition. Furthermore, hyper-osmolality resulted in **an** altered resting metabolism. There was a 12% decrease of ATP concentration, a 43% decrease in phosphocreatine and a 150% increase in creatine content compared with the control condition. There was also a 4-fold increase in tissue lactate. The authors suggested that the reduced ATP content demonstrated that the energy pathways of glycolysis and phosphocreatine were challenged to supply the cell with enough energy to meet its requirements during the 60 min hyper-osmotic condition, maybe in an effort to sustain cell volume. Additionally, increased lactate and creatine content could increase cellular osmolality and act as a compensatory mechanism to draw water back into the cell. Whilst the exact reason for the increase in resting metabolism could not be identified with certainty by the investigators, the reduction in the cell's energy charge (as described by the decreased ATP and high – energy phosphagen compounds, and the observed glycolysis, glycogenolysis and lactate production) could only negatively affect the muscles in an exercise environment.

Addressing the cell volume theory in whole body situation

Berneis et al. (1999) addressed the cell volume theory in humans by acute alterations of extracellular osmolality. The investigators induced either hypo – osmolality (intravenous administration of desmopressin 2 x 4 mg 12 hours apart, liberal water drinking, and infusion of hypotonic saline (0.4%)) or hyper-osmolality (intravenous infusion of hypertonic NaCl [2–5%; (wt/vol)] and restriction of water intake) and these changes were sustained during 17 h. In the hyper-osmotic condition, plasma osmolality was raised from 283 to 296 mosmol·kg⁻¹. In the hypo – osmotic condition, plasma osmolality decreased from 286 to 265 mosmol·kg⁻¹.

The authors suggested that these changes in plasma osmolality led to a modest state of cell shrinking or swelling. Using suitable tracers ($[1-^{13}\text{C}]$ leucine), leucine rates of appearance and disappearance were measured as indicators of protein metabolism and leucine oxidation as an indicator of catabolism. Glucose tracers ($[6,6-^2\text{H}_2]$ glucose) were used to monitor glucose kinetics and carbohydrate metabolism.

Hypo-osmolality resulted in a decrease of both leucine appearance (from 1.90 baseline to $1.79 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$), and leucine oxidation (from $0.34 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ baseline to $0.27 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) rates. Hyper-osmolality had no effect on leucine turnover, but led to an increase in the rate of glucose appearance ($12.4 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ vs. $11.2 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in iso-osmolality), indicating hepatic glucose production and increased plasma glucose concentrations ($5.1 \text{ mmol}\cdot\text{l}^{-1}$ vs. $4.9 \text{ mmol}\cdot\text{l}^{-1}$ in iso-osmolality, $p < 0.050$). The authors concluded that these effects were linked to modest cell swelling or shrinkages. Leucine release from endogenous proteins (representing protein breakdown) and leucine oxidation (indicating irreversible catabolism) were diminished during hypo-osmolality compared with iso-osmolality. Protein synthesis (non-oxidative leucine disappearance) during the hypo-osmotic condition was not altered, which indicates that net protein balance was improved during the hypo-osmolal state.

Hyper-osmolality and cell shrinkage led to increased hepatic glucose production (increased glycogenolysis), which resulted in increased plasma glucose concentrations. Their findings show in vivo that alteration of extracellular osmolality and body water exerts metabolic effects.

Keller et al. (2003) studied the effect of acute changes of extracellular osmolality on whole-body protein, glucose and lipid metabolism, using the same protocol as Berneis et al. (1999) to induce changes in extracellular osmolality.

During the experimental periods, plasma osmolality was $285 \text{ mosmol}\cdot\text{kg}^{-1}$ in the iso-osmotic condition, $296 \text{ mosmol}\cdot\text{kg}^{-1}$ in the hyper-osmotic condition and $265 \text{ mosmol}\cdot\text{kg}^{-1}$ in the hypo-osmotic condition. Their results showed that blood glucose concentration was higher ($5.1 \text{ mmol}\cdot\text{l}^{-1}$) during hyper-osmolality and lower ($4.7 \text{ mmol}\cdot\text{l}^{-1}$) during hypo-osmolality compared with iso-osmolality ($4.9 \text{ mmol}\cdot\text{l}^{-1}$).

Analysis of their results indicated a glucose sparing effect during hypo-osmolality and an increase in hepatic glucose production in the hyper-osmolality condition, which resulted in increased blood glucose concentration. Further measurements by indirect calorimetry showed that during hypo-osmolality, fat oxidation was higher and carbohydrate oxidation lower, supporting the glucose sparing effect of hypo-osmolality. Their findings on glucose metabolism agree with in vitro studies that glycogenolysis was reduced as a consequence of hypo-osmotic cell swelling (Haussinger et al., 1994; Stoll et al., 1992).

Data on protein metabolism showed that hypo- osmolality resulted in decreased leucine release from endogenous proteins (representing protein breakdown) and decreased leucine oxidation (representing irreversible catabolism). Leucine flux decreased from $1.90 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ at baseline to $1.79 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ during hypo-osmolality. Leucine oxidation decreased from $0.34 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ at baseline to $0.27 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ during hypo-osmolality. Protein synthesis was not altered during hypo-osmolality, and in conjunction with the decreased protein breakdown, hypo-osmolality resulted in a positive net protein balance.

Keller et al. (2003) also failed to find increased protein breakdown during hyper-osmolality, and they suggested that perhaps the increase in osmolality was insufficient to elicit significant protein catabolism. Overall, their findings tend to confirm in vivo the theory of Haussinger regarding cell shrinkage and protein breakdown. Despite the failure to find increased catabolism during hyper-osmolality, the investigations by the Berneis and the Keller group are the only studies addressing in vivo the cell volume theory on whole body metabolism. Still more research is required to address the cell volume theory in the whole body situation, especially in exercise environments. Whilst the Keller group used infusion of hypertonic solutions in a clinical environment, in an exercise environment it would be important to find a more feasible, practical and applicable method of inducing changes in extracellular osmolality. Ingestion of a highly hypertonic solution, such as glycerol, could provide the means for increasing extracellular osmolality and potentially altering cell hydration and volume.

Glycerol

Glycerol is a natural occurring trivalent alcohol that constitutes the backbone of the triglyceride molecule. Glycerol is a viscous, colourless, odourless liquid with a sweet taste (Nelson & Robergs, 2007). Physiological levels of blood glycerol are $0.03 - 0.5 \text{ mmol}\cdot\text{l}^{-1}$ (Gleeson et al., 1986; Nelson & Robergs, 2007) and it has been reported to be evenly distributed at low concentrations among all fluid compartments (intracellular and extracellular spaces), with the exception of the cerebral spinal fluid and aqueous humour (Lin, 1977). Glycerol ingestion in clinical settings has been used to combat intracranial and intraocular hypertension; by ingesting glycerol there is an increase in blood glycerol concentration that creates an osmotic gradient made possible by the blood brain barrier, water moves out of the intracranial and intraocular space into the blood, reducing the pressure in those areas (Lin, 1977).

In exercise settings, glycerol ingestion has been used as a means of hyperhydration. Ingesting a dosage of $1.0 - 1.2 \text{ g}\cdot\text{kg}^{-1}$ body mass raises blood glycerol concentration significantly to $12 - 15 \text{ mmol}\cdot\text{l}^{-1}$. As a result, plasma osmolality can rise by more than $10 \text{ mosmol}\cdot\text{kg}^{-1}$ (Freund et al., 1995; Gleeson et al., 1986; Montner et al., 1996). Since glycerol has the ability to retain water in the body, when glycerol ingestion is accompanied by large amounts of water (i.e., $20 \text{ ml}\cdot\text{kg}^{-1}$ body mass water), total body water increases (Nelson & Robergs, 2007; Robergs & Griffin, 1998).

In contrast, glycerol solutions of small volume (less than 700 ml total solution volume) have not been shown to increase total body water (Montner et al., 1996), but they still result in an increase in plasma volume (Gleeson et al., 1986; Montner et al., 1996). Gleeson et al. (1986) gave their participants a 400 ml glycerol solution to ingest, which contained $1 \text{ g}\cdot\text{kg}^{-1}$ body mass glycerol. The drinks' osmolality ranged between $1860 - 2350 \text{ mosmol}\cdot\text{kg}^{-1}$. Based on peak plasma glycerol values, and assuming that glycerol may be distributed throughout a fluid compartment equivalent to $0.65 \text{ l}\cdot\text{kg}^{-1}$ body mass, according to Wilmore and Costill

(1974), Gleeson et al. (1986) estimated that all orally ingested glycerol was absorbed within 30 min of ingestion. In the same study of Gleeson et al. (1986), plasma osmolality rose from 292 to 310 mosmol·kg⁻¹ and this was associated with an increase in plasma volume of approximately 10%. Since water moves between body water compartments due to osmotic forces, the authors suggested that the increase in plasma osmolality and circulation volume could have led to significant tissue dehydration.

In the above mentioned study by Gleeson et al. (1986) the authors used glycerol solutions in their aim to investigate whether glycerol could serve as an energy substrate during endurance cycling exercise. The reasoning for this was that glycerol is a gluconeogenic substrate that is converted into glucose in the liver (Bortz et al., 1972) and in a study by Terblanche et al. (1981) rats fed with glycerol were able to exercise for longer as glycerol was used as an energy substrate. Gleeson et al. (1986) showed that pre – exercise glycerol ingestion did not increase exercise time nor did it serve as a significant energy substrate during an endurance cycling protocol. The authors concluded that glycerol cannot be metabolised rapidly enough in humans in order to serve as a fuel source during strenuous exercise. This was also confirmed in a later study by Gleeson and Maughan (1988) when, following a 36-hour fast, the participants ingested a 400 ml glycerol solution (1 g·kg⁻¹ body mass) and then exercised at 70% VO₂max to exhaustion. Glycerol ingestion did not improve time to fatigue and results were similar to placebo.

Since glycerol is not a significant fuel source during exercise, and in small volumes it does not increase total body water significantly, it can be used solely as a means of increasing blood osmolality (Robergs & Griffin, 1998). Due to its osmotic properties, glycerol can potentially drive water into the extracellular space resulting in increases in plasma volume and tissue dehydration (Gleeson et al., 1986; Gleeson & Maughan, 1988) and can potentially provide the means for reducing myocyte hydration.

Another factor to be examined is the time course of this effect. Following peak blood glycerol concentration, there is a continuous decline as glycerol permeates

into the interstitial and intracellular space, including the skeletal muscles until it evenly distributes itself in the body's water compartments (Robergs & Griffin, 1998). Terblanche et al. (1981) showed that glycerol can be evenly distributed between the liver, blood and skeletal muscle, but that this takes about 1 to 2 hours after ingestion. Potentially, there could be a time window before distribution of glycerol in all water compartments where the alteration in body water distribution could allow the study of the cell volume theory and the effects of hyper-osmolality on muscle function during exercise in vivo.

Safety of glycerol ingestion

Participants ingesting glycerol solutions may have sensation of stomach fullness, light – headedness, headaches (Gleeson et al., 1986) nausea and vomiting (Murray et al., 1991). The sensation of fullness subsides within 15 – 20 min (Coutts et al., 2002).

Robergs and Griffin (1998) suggested that glycerol ingestion should be avoided by people with certain disorders. They suggested that as glycerol ingestion may lead to increased gluconeogenesis that takes place in the liver and results in elevated blood glucose, people with diabetes or liver disorders should not ingest glycerol. This, however, does not appear to hold true because while glycerol ingestion may lead to elevated blood glucose levels in rats (Terblanche et al., 1981), studies in man have shown that ingestion of a glycerol solution ($1 \text{ g} \cdot \text{kg}^{-1}$ body mass) does not increase resting blood glucose nor plasma insulin levels (Gleeson & Maughan, 1988; Gleeson et al., 1986).

Nevertheless, there are other clinical conditions that might be exacerbated following glycerol intake. Because glycerol may draw water from the intracranial space, which can lead to headaches, people with migraine or who tend to be susceptible to headache should probably not ingest glycerol. Individuals with renal problems should also seek medical advice before ingesting glycerol due to the increased role of the kidneys in glycerol removal when blood glycerol

concentrations are elevated. People with cardiovascular disorders should also refrain from ingesting glycerol as any resultant increases in plasma volume could lead to elevated blood pressure.

Overall, however, glycerol ingestion has been used widely in clinical settings and in doses between $1.0 - 1.5 \text{ g}\cdot\text{kg}^{-1}$ body mass and there seems to be no scientific justification for a deleterious clinical consequence resulting from such intakes (Robergs & Griffin, 1998).

Potential effects of glycerol ingestion on body water distribution and resultant effects on exercise

As indicated from the findings of Gleeson et al. (1986) and Gleeson and Maughan (1988), ingestion of a concentrated glycerol solution (400 ml, $1 \text{ g}\cdot\text{kg}^{-1}$ body mass glycerol) results in plasma and blood volume increases and there is potentially a degree of tissue dehydration. If the in vitro findings from the studies described Haussinger and his group apply in a whole body situation, reduction in muscle water could have an adverse effect on muscle metabolism (i.e., reduced ATP and phosphocreatine content, reduced protein synthesis) and function (reduced contractility), which could affect exercise performance and the outcome of strength training. On the other hand, increases in blood volume could have a positive effect on aerobic exercise; increase in blood volume results in greater cardiac output that may result in increased maximum oxygen uptake ($\text{VO}_{2\text{max}}$) and aerobic exercise performance (Coyle et al., 1986). Further information on both muscle dehydration and increase in blood volume and their effect on exercise are presented below.

Muscle dehydration and exercise

In vivo evidence regarding muscle function and metabolism in a dehydrated state comes from studies where the participants performed resistance exercise in a dehydrated state (implying muscle hydration was also reduced), which was expressed as reduced total body water.

Body hydration and strength exercise

Acute effects of body dehydration on strength exercise

Studies investigating the effects of dehydration (expressed as reduced total body water) on strength have reached conflicting results, reporting either a decrease (Bosco et al., 1974; Bosco et al., 1968; Schoffstall et al., 2001; Torranin et al., 1979; Viitasalo et al., 1987) or no change (Bigard et al., 2001; Greenleaf et al., 1967; Greiwe et al., 1998; Montain et al., 1998) in power output. This could be attributed either to the degree of dehydration, which ranged between 1.7% (Schoffstall et al., 2001) to 5.7% (Bosco et al., 1974) body mass, or the method used to elicit dehydration or the method used to measure strength (isometric or dynamic model of exercise or vertical jump). Available methods to induce dehydration are the use of a sauna (passive dehydration), vigorous exercise (active dehydration), the use of diuretics, or fluid intake restriction. Active dehydration may lead to fatigue or depletion of glycogen, which will impair performance independent of the dehydration effects. In the above referenced studies, no vigorous exercise was used. Sauna exposure also increases core temperature; exercising under elevated muscle and core temperature limits work capacity and promotes fatigue (Galloway & Maughan, 1997; Nielsen et al., 1981) and may contribute to fatigue during strength exercise (Barr, 1999). Torranin et al. (1979) showed that isometric endurance decreased following sauna exposure and 4% dehydration. Viitasalo et al. (1987) reported decreased maximal isometric leg strength (7.8%) following exposure to sauna. Schoffstall et al. (2001) stated that

passive dehydration resulting in 1.5% body mass dehydration decreased maximum bench press performance. Greiwe et al. (1998) described no change in isometric knee extension peak torque or in time to fatigue following 3.8% body mass dehydration (sauna exposure).

Montain et al. (1998) used magnetic resonance spectroscopy (^{31}P -MRS) to examine the effects of hypohydration on muscle metabolites and performance. After a 4% loss in body mass following active dehydration, the participants performed single – leg knee extensions. Maximal voluntary contraction (MVC) was not affected, but endurance (time to fatigue) was impaired and reduced by 15%. These authors had hypothesized that because elevated muscle H^+ and Pi concentrations reduce muscle force production during repeated contractions (Fitts, 1994), and dehydration was likely to increase H^+ and Pi relative concentrations, significant losses of body water would reduce muscle metabolism and strength performance. However, their data showed that there was not any difference in muscle metabolism between the euhydrated and dehydrated state. These results are in contrast to those of Antolic et al. (2007) who found reduced ATP and phosphocreatine content in skeletal muscle cells following osmotic dehydration. In a thorough review of literature, Judelson et al. (2007a) concluded that hypohydration appears to negatively influence muscular strength, power and high – intensity endurance in terms of performance. Beyond the acute effects, one should also consider the effects of chronic muscle dehydration. Resistance exercise is usually carried out with the purpose of increasing strength and muscle size. Muscle protein synthesis takes place in the recovery period. If dehydration persists during recovery and muscle hydration is reduced (thereby decreasing protein synthesis according to the cell volume theory), it could potentially minimise the expected training effect.

Resistance exercise training and hypertrophy

Improvement in muscle strength and hypertrophy appear following effective regular resistance training. Strength can increase after only a few days of training due to neuromuscular adaptations (Del Balso & Cafarelli, 2007; Folland &

Williams, 2007), but hypertrophy and further gains in strength result after about 8 or more weeks of training (Folland & Williams, 2007; Garfinkel & Cafarelli, 1992; Young et al., 1983). Gains in muscle size and strength are elicited by training programmes using high loading at intensities greater than 75% max of 1 RM (Kanehisa et al., 2002). Training at intensities lower than 50% max of 1 RM does not seem to induce any hypertrophy (Moss et al., 1997). Training volume is also important; resistance exercise programmes designed to elicit hypertrophy usually involve 3 sessions **per** week, and workloads consisting of 3 – 5 sets of 8 – 10 repetitions (McCaulley et al., 2009; Schott et al., 1995; Smith & Rutherford, 1995; Yamamoto et al., 2008).

In terms of hypertrophy, the adaptive response reflects the overall summation of protein metabolism after exposure to multiple bouts of resistance exercise. Resistance exercise increases both protein synthesis and breakdown, but in the post-absorptive state, muscle protein synthesis exceeds protein breakdown (Mougios, 2006). A positive protein balance (synthesis > breakdown) results in an increase in muscle cross sectional area, which enhances force generation (Maughan, 1984a).

The induced skeletal muscle hypertrophy produced by strength exercise has been linked to acute changes in the cellular processes involved in protein turnover and muscle growth (Crewther et al., 2006; Tipton & Wolfe, 2001) as hormones and growth factors interact to regulate muscle protein remodelling following resistance exercise (Kraemer et al., 1998). Testosterone, growth hormone, insulin, and insulin-like growth factor (IGF-1) are known to promote increases in muscle protein synthesis (Baar, 2006; Tipton **&** Wolfe, 2001).

Kraemer et al. (1998) investigated the hormonal responses following resistance exercise, which consisted of four sets of ten repetitions of four different exercises (16 sets total). Their findings showed that circulating testosterone levels increased acutely following the exercise. Hakkinen **and** Pakarinen (1993) demonstrated a similar effect following a session consisting of 10 sets of 10 repetitions at 10 RM, in a squatting exercise.

Growth hormone is another potent anabolic hormone influencing muscle tissue growth by increasing protein synthesis and reducing the degradation of muscle protein (Widerman et al., 2002). Heavy resistance exercise at high intensity (above 70% of 1RM), similar to protocols inducing hypertrophy, seems to result in a greater growth hormone response (Kraemer et al., 1990). The anabolic action of growth hormone is then mediated by the effect of secondary proteins known as somatomedins or insulin-like growth factors (IGFs) with IGF –1 being the most important (Kraemer et al., 1990; Crewther et al., 2006).

Insulin's main role is to regulate blood glucose, by increasing glucose uptake by the tissues when glucose levels in the blood rise. Additionally, insulin increases the uptake of amino acids into the muscle cell (Mougios, 2006). Amino acid availability is essential for protein synthesis in the recovery period following resistance exercise, and insulin in humans inhibits protein breakdown (Rooyackers & Nair, 1997); therefore insulin is considered an anabolic hormone.

Cortisol is considered the primary catabolic hormone as it decreases protein synthesis and increases protein degradation. Cortisol levels in the blood rise following resistance exercise, especially following a heavy workload of many sets at high intensity (Kraemer & Ratamess, 2005). It is of interest to note that the same type of training that elicits hypertrophy results in greater acute increase of both the anabolic hormones and cortisol.

Hypohydration and its effects on hormonal responses to resistance exercise

Judelson et al. (2008) studied the effect of hydration state on the hormonal and metabolic responses to resistance exercise. Seven male participants performed 6 sets of 10 repetitions of back squats at 80% of 1 RM on three different occasions: in a euhydrated state, under 2.5% body mass hypohydration and 5.0% hypohydration. The hypohydrated states were elicited by fluid restriction and a low-intensity walking exercise on a treadmill in a heated environmental chamber (36 – 37°C, 40 – 50% relative humidity), the day before the resistance exercise protocol. Following completion of exercise sessions, there were different hormonal responses between the euhydrated and dehydrated conditions. The

concentrations of the catabolic stress hormones, cortisol and epinephrine, in the recovery phase in the 5% hypohydration condition were higher than under the other conditions, and the epinephrine area under the curve tended to be increased. The area under the curve of testosterone, an anabolic hormone, was decreased in the 5% hypohydrated compared with the euhydrated condition. Insulin and glucose circulating levels were increased in both the hypohydrated conditions, but the investigators suggested that this might be due to an increased insulin resistance due to hypohydration and a decrease in muscle cell volume (Schliess & Haussinger, 2000). IGF-1 concentration was similar across conditions and there was no difference in growth hormone levels between conditions.

The investigators concluded that hypohydration strongly enhanced a catabolic hormonal response to resistance exercise and attenuated the anabolic response to resistance exercise, producing a less beneficial muscle growth post-exercise milieu. Long-term exercise under a muscle-dehydrated state could negatively affect the improvements in strength that are expected from resistance exercise training.

Increased plasma and blood volume and potential effects on aerobic exercise

Maximum oxygen uptake ($\text{VO}_{2\text{max}}$) is defined as the highest rate at which oxygen can be taken up by the body and utilised during severe exercise. The current belief is that maximum cardiac output is the principal limiting factor for $\text{VO}_{2\text{max}}$ during cycling tests, as the observed normal range of $\text{VO}_{2\text{max}}$ in trained men and women of similar age is due principally to variation in maximal stroke volume (Bassett & Howley, 2000; Ekblom, 2000; Gonzalez-Alonso & Calbet, 2003). Stroke volume can increase due to greater blood volume (Hopper et al., 1988; Gledhill, 1985; Kanstrup & Ekblom, 1982) because of the Frank-Starling mechanism (Coyle et al., 1990; Ekblom, 2000; Fortney et al., 1981; Kanstrup & Ekblom, 1982) as increased ventricular filling pressure leads to increased output

(stroke volume). The increased stroke volume during exercise when plasma and blood volume are elevated can be explained by a concomitantly increased end – diastolic volume and reduced end–systolic volume (Kanstrup & Ekblom, 1982). Vanoverschelde et al. (1993) studied the relationship between left ventricular diastolic function and exercise capacity in normal participants by assessing left ventricular systolic and diastolic function in both sedentary and trained athlete groups. Some of the sedentary individuals had a VO_2max close to the athletic group. These individuals with a higher VO_2max had a higher end-diastolic volume and faster peak early filling velocities. Early diastolic filling was the most powerful independent correlate of VO_2max and the authors concluded that the diastolic properties of the heart play a central role in determining exercise capacity in both trained and untrained individuals and that this increased ventricular filling could be a result of elevated venous return.

Endurance trained individuals exhibit a greater blood volume and total haemoglobin levels than sedentary individuals (Kjellberg et al., 1949). A major adaptation to endurance training is an increase in stroke volume resulting from an increase in circulating blood levels (Coyle et al., 1986; Clausen, 1977) attributed to increases in plasma volume alone. During endurance training, for example, plasma volume has been shown to increase by $4.5 \text{ ml}\cdot\text{kg}^{-1}$ body mass (10%), even 24 hours after a single high-intensity (85% VO_2max) cycling session (Gillen et al., 1991). Convertino et al. (1980) showed that following 8 days of a cycling training protocol at 65% VO_2max , a blood volume increase resulted, which was due to a 427 ml increase in plasma volume. In a similar study by Green et al. (1990), the participants exercised at 65% VO_2max for 3 consecutive days. The results showed that plasma volume increased by 624 ml, which resulted in improved stroke volume and cardiac output. In both studies, red cell volume remained unchanged. This training-induced hypervolaemia is quickly lost when training is stopped (Convertino et al., 1980). Coyle et al. (1986) observed that 2 to 4 weeks of detraining led to a 9% decrease in blood volume, which was associated with a 12% decrease in stroke volume and a 6% reduction in VO_2max . In the same study, when blood volume was expanded to a similar level as in the trained state

(via the infusion of a 6% dextran solution), the effects of detraining on cardiovascular response were reversed and VO_2max returned to values not different from the trained values.

In laboratory settings, plasma volume expansion (independent of increases in haemoglobin and haematocrit) leading to expansion of blood volume has been shown to increase VO_2max , especially in untrained participants who do not have an elevated blood volume as a result of endurance training (Coyle et al., 1990; Coyle et al., 1986; Krip et al., 1997). Coyle et al. (1990) infused a 6% dextran solution to expand plasma volume in untrained individuals. Firstly, they expanded plasma volume by 282 ml (7.3%), which resulted in a 4.5% increase in blood volume and a higher stroke volume during exercise. Following plasma volume expansion, the participants exercised on a treadmill at maximal intensity to exhaustion. VO_2max during this test was significantly increased by 3.7% and time to fatigue increased by 6.8% compared with the normal values before plasma expansion (9.4 vs. 8.8 min). Further expansion of plasma volume by 500 – 600 ml (18%) above normal values did not result in any improvements in performance, as VO_2max during exercise and time to fatigue was similar to the normal value prior to plasma volume expansion. This outcome was attributed to the fact that the large increase in plasma volume resulted in dilution of the haemoglobin concentration by 11.4% that limited the blood's oxygen carrying capacity, while the increase in blood volume could not offset the haemodilution. In a similar study, Krip et al. (1997) infused 500 ml of 6% Dextran to untrained participants. This elicited increases in stroke volume and cardiac output during exercise, and VO_2max increased by 7%.

As already suggested, large plasma volume expansions may lead to significant dilution of haemoglobin concentration, as the number of red blood cells remains unchanged following increases of plasma water, and this reduces the oxygen carrying capacity of the blood (Berger et al., 2006; Coyle et al., 1990; Hopper et al., 1988). However, plasma volume can be expanded to an optimal level that can offset the effects of modest haemodilution and the improvement in stroke volume can result in positive effects on aerobic performance (Coyle et al., 1986; Coyle et

al., 1990; Hopper et al., 1988; Krip et al., 1997). Despite the fact that some researchers reported no changes in VO_2max following acute plasma volume expansion (Kanstrup & Ekblom, 1982; Kanstrup & Ekblom, 1984; Mier et al., 1996) recent data from Berger et al. (2006) also showed an increase in $\text{VO}_{2\text{peak}}$ and time to exhaustion during severe-intensity exercise following induction of hypervolaemia.

Hence, apart from increases in VO_2max , plasma volume expansion may elicit performance improvements. Luetkemeier and Thomas (1994) investigated the effects of acute plasma volume expansion (infusion of 400 ml of a 6% dextran solution) on a simulated cycling time trial. Plasma volume was expanded by 12.4%. The results showed that average power for the entire time trial was significantly higher compared with the control condition (250 W vs. 215 W), which resulted in better performance times (81 min in hypervolaemia vs. 91 min in control condition). In another study, Berger et al. (2006) infused an average of 588 ml of a plasma expander (Gelofusine, 4% succinylated fluid gelatin; $7 \text{ ml} \cdot \text{kg}^{-1}$ body mass) resulting in a 14% increase in plasma volume. The participants exercised to exhaustion at a high intensity, which was set at 70% of the difference between the gas exchange threshold and VO_2max . Time to fatigue increased following plasma expansion (424 s) compared with the control condition (365 s). Also, $\text{VO}_{2\text{peak}}$ attained at the end of exercise was higher following plasma expansion ($4.12 \text{ l} \cdot \text{min}^{-1}$) than in the control condition ($3.9 \text{ l} \cdot \text{min}^{-1}$).

Conclusions on blood volume expansion

Without doubt, blood volume plays a major role in cardiovascular function and affects aerobic exercise. Endurance training results in increases in blood volume even within the first few days. Acute plasma volume expansion via infusion of plasma expanders may function as an ergogenic aid for untrained individuals who do not already possess a high blood volume.

Plasma volume expansion must be within a certain limit in order to elicit improvements in VO_2max or exercise performance. Small volumes (generally 200 – 300 ml, and as much as 500 ml maximally) have been shown to offer a

beneficial effect. Larger increases in plasma volume with unchanged red blood cell number lead to dilution of haemoglobin concentration and reduce the blood's oxygen carrying capacity.

Rationale of the experimental investigations of this thesis

Concentrated glycerol solutions can be used for the purpose of elevating blood osmolality (Robergs & Griffin, 1998), as glycerol is not a major substrate for energy during exercise and it does not affect resting blood glucose or plasma insulin (Gleeson et al., 1986). If a hypertonic solution is added to extracellular fluid, extracellular osmolality increases and causes movement of water out of the cell into the extracellular compartment. The net effect is an increase in extracellular volume, a decrease in intracellular volume, and a rise in osmolality in both compartments (Guyton & Hall, 1996). Therefore glycerol ingestion may provide the means for indirectly addressing the cell volume theory by reducing muscle hydration.

Reduction in muscle hydration could have an acute or even a chronic effect. In vitro, exposure of muscles to a hypertonic glycerol solution has been shown to result in decreased twitch tension (Caputo, 1968). Judelson et al. (2007b) suggested that when individuals exercise in a hypohydrated state, it negatively affects the action of anabolic hormones such as testosterone and insulin whose physiological action promotes muscle growth and hypertrophy following strength training. Increases in muscle strength and muscle size appear after a lengthy training period. If athletes exercise in altered muscle hydration state during the training period, the training outcome could be affected.

Glycerol ingestion ($1 \text{ g} \cdot \text{kg}^{-1}$ body mass in a 400 ml solution) has been shown to increase plasma volume by approximately 10% (Gleeson et al., 1986; Gleeson & Maughan, 1988). This level of plasma volume expansion, produced by glycerol, is similar to that reported to be the optimal level of expansion that results in positive effects on stroke volume and aerobic exercise (Berger et al., 2006; Coyle et al.,

1986; Coyle et al., 1990; Hopper et al., 1988; Warburton et al., 1999). It would be of interest to examine whether this resultant change in plasma volume would have any beneficial effect on VO_2max , especially bearing in mind that apart from increasing plasma volume, glycerol ingestion could well decrease muscle cell hydration.

Before venturing into investigating these theories, it would be important to investigate the potential changes in body water distribution following ingestion of a small-volume highly-hyper-osmotic glycerol solution and to establish a time course of any such effects.

Chapter 2

General Methods

Ethical Approval

Prior to the start of each of the experiments, ethical approval was obtained from the Loughborough University Advisory Committee, as all five studies described in this thesis involved human volunteers. All participants were healthy male adults over 18 years of age. Before signing a consent form, all volunteers were fully informed of procedural details of the experiments, both orally and in writing. They were also reminded of their right to withdraw from the studies at any point, without obligation to provide any reason for doing so.

Standardisation prior to experimental trials

All trials took place in the morning after an overnight fast and trials were separated by at least 7 days. Participants were asked to maintain the same nutritional and activity patterns during the 24 hours prior to the start of each experimental trial. Trials were randomised using a Latin square design.

Blood sampling and analysis

Blood samples were collected during all studies described in this thesis. All analytical procedures are described below and the specific intervals and volume of blood sampling are described in detail in the methods section of each experimental chapter.

In all experimental chapters, for the purpose of blood sampling, one arm was warmed up by immersion in hot water (42° C) for 10 min. Then an indwelling canula (21 g, butterfly) was inserted into a forearm vein, and remained in place throughout the session, to allow the withdrawal of multiple blood samples. In the rare case where the arm 'got a little cold' during the session, the arm was placed on the subject's legs (use of body heat) and towels were used to cover the arm in

order to keep it sufficiently warm to allow good blood flow and to reduce problems in blood sampling.

Two resting blood samples were drawn 10 min apart, in all experimental chapters described in this thesis. Statistical analysis confirmed that in each study there was no difference in measured parameters between the two resting samples, and for standardization all 'Rest' samples mentioned in this thesis refer to the second resting blood sample, which was drawn immediately before ingestion of the various solutions.

Haemoglobin concentration and haematocrit

Whole blood samples (4 ml) were collected in a syringe and 2.5 ml was then dispensed into a K₂EDTA containing tube and 1.5 ml into a plain plastic tube. Haemoglobin concentration was determined using the cyanmethaemoglobin method and haematocrit was determined by microcentrifugation (Hawksley Micro-Haematocrit Centrifuge, U.K.) using the anticoagulated blood samples. Haemoglobin concentrations were determined in duplicate while haematocrit was measured in triplicate. No corrections were made for trapped plasma.

Percentage changes in blood volume, red cell volume and plasma volume were calculated using the formulae described by Dill and Costill (1974) using changes in haemoglobin concentration and relative volume of red blood cells to estimate percentage changes ($\Delta\%$) in blood, plasma and red blood cells volume. In the present thesis there was no direct measurement of blood, plasma or red cell volume. Dill and Costill equations provide only an estimation of percentage changes in blood, plasma and red blood cells volume over time based on changes in haemoglobin concentration and haematocrit, as opposed to other methods that use bromide or inulin tracers and measure extracellular water directly. Other methods that determine alterations in plasma volume incorporate changes in circulating protein and albumin. The method of Dill and Costill was more appropriate to use in the current studies due to the fact that haemoglobin is trapped in the red blood cells and will not leave the vascular compartment,

whereas proteins can enter and leave the vascular compartment during exercise (Harrison, 1985), which may give unreliable results.

The equations used in this thesis to monitor changes in blood and plasma volume during different modes of exercise according to Dill and Costill are described below:

$$\Delta BV \% = 100 (BV_2 - BV_1) / BV_1 \quad BV \text{ is blood volume}$$

$$\Delta CV \% = 100 (CV_2 - CV_1) / CV_1 \quad CV \text{ is red blood cells volume}$$

$$\Delta PV \% = 100 (PV_2 - PV_1) / PV_1 \quad PV \text{ is plasma volume}$$

Numbers 1 and 2 indicate different sampling time; sample 2 is taken after sample 1. In all the experimental chapters described in this thesis, sample 1 was the resting blood sample before ingestion of any solution, and all subsequent blood samples were compared against the resting blood sample.

Blood glucose and lactate

Following collection of whole blood samples, duplicate aliquots of 100 μl from the K_2EDTA tube were immediately deproteinised in 1 ml of ice cold $0.3 \text{ mol}\cdot\text{l}^{-1}$ perchloric acid. Following centrifugation, the supernatant was used for measurement of glucose and lactate. Glucose was determined using the GOD-PAP method (Randox) method (glucose standard was $5.5 \text{ mmol}\cdot\text{l}^{-1}$) and lactate concentration was determined fluorimetrically, according to Maughan (1982). For lactate measurements, resting blood samples were analyzed separately from samples obtained during exercise. For lactate measurements, resting blood samples were analyzed separately from samples obtained during exercise. The lactate standards used for resting samples were 0, 1.0, 2.5 and $5.0 \text{ mmol}\cdot\text{l}^{-1}$, and for samples drawn during exercise, they were 0, 2.5, 5.0, 10.0, 15.0, and $20.0 \text{ mmol}\cdot\text{l}^{-1}$. Both glucose and lactate samples were determined in duplicate.

Serum osmolality, glycerol and electrolyte concentrations

The whole blood samples collected into plain tubes were centrifuged at 1500 g for 15 minutes at 4°C (ALC Multispeed Refrigerated Centrifuge, U.K.) and the serum was collected. Duplicate aliquots of 20 µl serum were deproteinised in 200 µl ice cold 0.3 mol·l⁻¹ perchloric acid. Following centrifugation, they were stored at approximately -20 °C and were later fluorimetrically analysed for glycerol according to Boobis and Maughan (1983). Remaining serum samples were then stored in the refrigerator at approximately <4°C until analysed for osmolality (within 14 days of sample collection). Serum osmolality was measured by freezing point depression (Gonotec Osmomat 030 Cryoscopic Osmometer; Gonotec, Berlin, Germany). Serum osmolality and glycerol were measured in all experimental studies.

In the final experimental study, described in chapter 7, serum sodium and potassium concentration were also measured using flame photometry (Corning Clinical Flame Photometry 410C; Corning Ltd., Halstead, Essex, U.K.).

Isometric knee extension – maximum voluntary contraction (MVC)

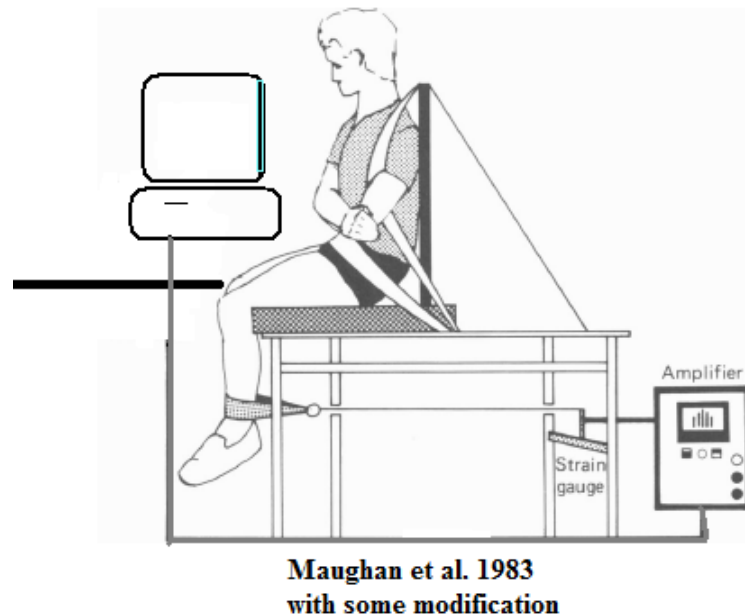
Isometric knee extension was used in the study described in chapter 4.

Participants performed single-leg isometric knee extensions on a pre-calibrated isometric dynamometer on both legs. Calibration was carried out before each trial by applying a range of known weights, 0 – 80 kg. Maximal calibration value was higher than each participant's maximal voluntary contraction.

Each participant was seated and strapped on an isometric chair (Fig 2.1.) according to Maughan et al. (1983). The isometric chair had a moveable back, which allowed the seat to be adjusted so that the back of the subject's knee was at the front edge of the isometric chair and the participant's back was in an upright position. Each participant was strapped on to the isometric chair with an adjustable harness, which prevented extension of the hip when the participant performed quadriceps activations. Hip and knee joints were flexed at ~90°. During exercise, the participants held their arms crossed over their chest. An inelastic

cable was attached to the exercising leg just above the ankle joint on one end and to a strain gauge on the other end. Each participant tried to extend the knee and the force generated was measured by the strain gauge. The analogue signal from the gauge was amplified, digitised and transmitted to a computer where a graph of the produced force was displayed on a monitor in real time, such that the participants had visual feedback of the force they were producing. The maximum voluntary contraction (MVC) test protocol consisted of three sets of 3 s maximal muscle activations, with 1 min of recovery in-between. All three sets were executed on the same leg before switching to the other leg.

Figure 2.1 Isometric knee extension. Isometric chair set up.

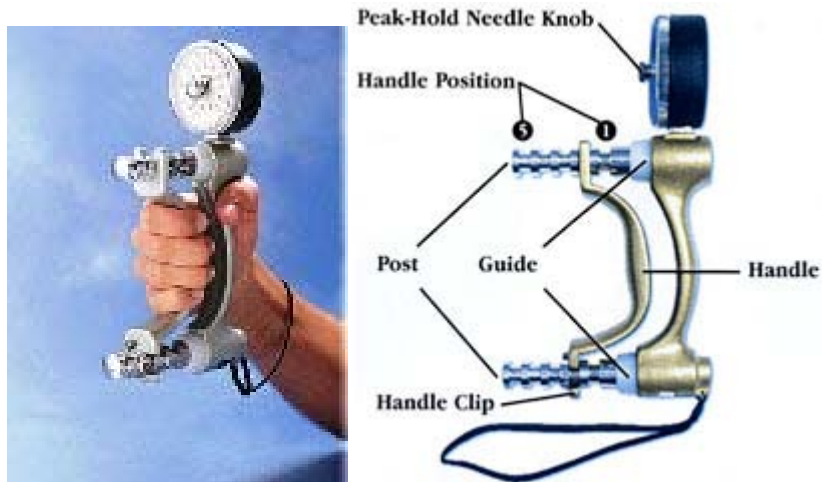


Handgrip strength

Handgrip strength was determined in the study described in chapter 5, using a Jamar Dynamometer (Lafayette Instrument, Germany; Fig 2.2). Participants exercised while seated and the exercising arm was extended by the side of the body. Maximum force was taken as the highest value obtained from three

attempts separated by 1 min of rest. All three efforts were executed on the same arm before switching to the other arm.

Figure 2.2 Jamar Dynamometer



Forearm muscle volume

Forearm muscle volume was indirectly estimated by water displacement according to Maughan et al. (1984b). The arm of the volunteer was slowly submerged into a volumeter (eureka can) full of water and the displaced water was collected into a jug and weighed on a kitchen scale with accuracy of 1 g. As the volume of the displaced water is equal to the volume of the submerged forearm, forearm volume can be simply derived. The forearm was taken to extend from the olecranon process to the ulnar styloid. Forearm muscle volume was estimated using the equations by Maughan et al. (1983):

$$\text{Forearm fat content (\%)} = (2.80 \times \text{biceps skinfold, mm}) + 2.7$$

$$\text{Muscle volume (ml)} = (0.92 \times \text{total forearm volume}) - (0.97 \times \text{fat volume}) - 38$$

Bicep skinfold was measured in triplicate using a Harpenden Skinfold Calliper (Harpenden Callipers, U.K.).

This method was used in the study described in chapter 5.

Maximal oxygen uptake test VO_2max

Measurement of maximal oxygen uptake was performed in the last two studies described in chapters 6 and 7. In both studies, participants exercised on an electronically-braked cycle ergometer (Gould, Bilthoven, The Netherlands). Participants cycled at a self-selected cadence at or above 60 rpm. Expired air samples were collected in a Douglas bag during the final 1 minute of each stage before determination of oxygen and carbon dioxide content (Servomex 1400, Crawley, East Sussex, U.K.), gas volume (Harvard Dry Gas Meter, Harvard Apparatus Ltd., Kent, U.K.) and gas temperature (Edale Digital Thermometer). In the study described in chapter 6, VO_2max was determined using a continuous graded exercise protocol. Initial workload was set at 100 W and this increased by 50 W every 3 min until the participants reached volitional exhaustion. During the last stages of a test, if believed that the participant would not be able to tolerate a greater increase in workload (heart rate within 10 beats of age-predicted maximum heart rate, and/or rating of perceived exertion 19-20), the workload was increased by only 25 W. The test ended when the participant could not maintain a cadence above 60 rpm.

In the final experimental study, described in chapter 7, VO_2max was assessed using a discontinuous protocol. The purpose was not only to measure VO_2max , but also to firmly establish the intensity at VO_2max that would be used later in the main exercise protocol of the study. Cycling stages, intensity and increases in workload were similar to those mentioned above, with the only exception being the duration of the first stage, which was 5 min.

Coefficient of variation for analytical procedures

Coefficients of variation (CV) for each of the analytical procedures described are shown in table 2.1. CV was calculated as the standard deviation of the difference between duplicates and expressed as a percentage of the mean value obtained for samples produced throughout this thesis.

Table 2.1 Mean, SD and Coefficient of variation (%) of duplicates obtained for analytical procedures conducted throughout this thesis.

Assay	n	Mean	SD	%CV
Haemoglobin (g·l ⁻¹)	30	141	12	1.6
Haematocrit (%)	30	45	3	1.1
Blood glucose (mmol·l ⁻¹)	30	6.0	1.0	0.8
Blood lactate (mmol·l ⁻¹)	30	5.0	4.0	1.1
Serum osmolality (mosmol·kg ⁻¹)	30	294	10	0.7
Serum glycerol (mmol·l ⁻¹)	30	5.0	5.0	1.1
Serum potassium (mmol·l ⁻¹)	30	4.8	0.6	1.3
Serum sodium (mmol·l ⁻¹)	30	142	2	0.7
Forearm volume (ml)	30	1060	93	1.4
Handgrip strength (kg)	30	44	10	0.8

Statistical analysis

Specific details regarding the statistical procedures used during each experimental trial are described in the methods section of each experimental chapter. All data sets were checked for normative distribution using the Shapiro-Wilks test. When data were not normally distributed, they were analysed using non-parametric tests. All data were analyzed using SPSS (versions 14 and later) and Minitab version 15, Minitab Ltd., Coventry, UK, statistical software.

In general, all studies in this thesis had a cross-over design. In chapters 3, 4 and 5, data were subjected to a two-factor repeated measures analysis of variance. Main effects of time and trial are reported, however, appropriate post-hoc tests were employed only when an interaction effect was observed. In chapters 6 and 7, where there were only two main experimental trials, the results were compared using paired t-tests. Significance level was set at $P < 0.050$.

Chapter 3

Acute effects of glycerol, glucose and water
ingestion on body water distribution

Introduction

Body hydration is an important health factor as many physiological functions such as the cardiovascular and thermoregulation functions are affected by an individual's hydration status (Maughan, 2003). Water accounts for approximately 50 – 60% of the human body (35 – 42 l for an average 70 kg person). Body water is distributed in the intracellular space (two thirds of total body water) and the extracellular space. The extracellular space is divided into the interstitial space (15% total body mass) and the plasma (5% total body mass) (Guyton & Hall, 1996).

Cellular hydration state may play a role in cell metabolism and function. Based on a theory first suggested by Haussinger (Haussinger et al., 1994; Lang et al., 1998), when cells shrink (i.e., high extracellular osmolality, dehydration) metabolism becomes mainly catabolic and when cells swell, metabolism becomes anabolic. Even small fluctuations in cell hydration and cell volume are thought to act as a separate and potent signal for cellular metabolism (Haussinger, 1996). Cell swelling has been shown to inhibit proteolysis in hepatic cells (Hallbrucker et al., 1991a,b; Haussinger et al., 1991; Haussinger et al., 1990a,b), whereas cell shrinkage inhibits protein synthesis and stimulates proteolysis (Meijer et al., 1993; Stoll et al., 1992). Furthermore, glycogen synthesis can be stimulated by exposure of hepatocytes (Baquet et al., 1990; Meijer et al., 1992) and muscle cells (Low et al., 1996) to a hypo-osmotic environment, whereas cell shrinkage following exposure to a hyper-osmotic milieu decreases glycogen synthesis (Baquet et al., 1990; Haussinger, 1996). If the cell volume theory applies in a whole body situation, in an exercising environment, where there is loss of body water, and reductions in intracellular water could hinder recovery and performance in subsequent exercise bouts or sessions. Studies involving animals and humans showed that cell shrinkage signals the cleavage of glycogen, lysing of proteins and a temporary halt to the formation of both glycogen and protein (Ritz et al., 2003). In a study by Keller et al (2003), acute hyper-osmolality (296 mosmol·kg⁻¹) via infusion of hypertonic NaCl solution over a 17 – hour period on metabolism

resulted in an increased plasma glucose concentration as a result of increased hepatic glycogenolysis and glucose production; whereas acute hypo-osmolality ($265 \text{ mosmol}\cdot\text{kg}^{-1}$), induced via a combination of intravenous desmopressin and 2.4 l/12 h water intake, resulted in decreased plasma glucose concentration indicating reduced hepatic glucose production. Their findings supported in general the cell volume hypothesis in an in vivo model. Therefore, acute changes in extracellular osmolality may play a role in metabolic regulation.

It is still unknown whether ingestion of hypertonic solutions resulting in elevated extracellular osmolality, produces any real effect on body water distribution that may affect cell hydration and metabolism during rest or exercise. Ingestion of high osmolality glucose drinks has been shown to cause a decrease in plasma volume as hypertonicity of the beverages resulted in the movement of water from the body water pool into the intestinal lumen, causing a transient decrease in the effective body water content (Merson et al. 2002). Glycerol, on the other hand, has been used in solutions for hydration and over-hydration purposes prior to exercise as a means of attenuating the effects of dehydration that occurs during aerobic exercise (Wagner, 1999), as reductions in blood volume cause a decline in cardiovascular function, maximum oxygen uptake and time to fatigue (Coyle et al., 1986). An increase in the blood glycerol concentration can result in an increase in osmotic forces thereby promoting fluid retention in the vascular space when large volumes of water ($\sim 2 \text{ l}$) are ingested (Freund et al., 1995). Total body water increases and this over-hydrated state has been shown to improve endurance performance (Anderson et al. 2001; Hitchins et al., 1999; Montner et al., 1996) as it may delay the onset of dehydration and give a performance advantage compared with someone perhaps already hypohydrated (Nelson & Robergs, 2007). Overall, research findings on the effects of glycerol hyperhydration are equivocal as some studies failed to find an ergogenic effect of glycerol hyperhydration during aerobic exercise. This may be due to discrepancies in glycerol dosage, timing of ingestion, environmental temperature, humidity and exercise intensity (review by van Rosendal et al., 2010). On the negative side, it is possible that glycerol ingestion in smaller volumes and more concentrated

solutions may lead to a different osmotic effect on body water distribution. High increases in glycerol blood concentration could create a high extracellular osmolality and a concentration gradient that may lead to water shifts into the extracellular space favouring cellular dehydration and tissue shrinkage (Maughan, 1998).

Because of the possible water shifts caused by ingestion of hypertonic drinks and the effects this could have on the effective body water content and cellular hydration, the purpose of this study was to examine the consequences of water, glucose and glycerol ingestion on body water distribution during rest, and to establish a time course of any effects.

Materials and Methods

Following ethics approval by the Loughborough University Ethical Advisory Committee, nine healthy male volunteers participated in this study. After a written and verbal explanation of all procedures, and their right to withdraw at any point, each subject signed a written consent form.

Participants had a mean (\pm SD) age of 24 ± 3 years, height of 177 ± 5 cm and body mass of 74.8 ± 10.8 kg. Experimental trials were separated by at least 7 days and took place at the same time of the morning, after an overnight fast. Subjects followed similar nutritional and physical activity patterns for the 24 – hour period preceding each trial.

Each subject participated in three experimental trials in randomised order, which was assigned using a Latin square design.

Experimental procedure

Upon arrival at the laboratory, the subjects voided and their body mass was determined wearing minimal clothing using a beam balance (Marsdens type 150, Marsdens Weighing Machines, London, U.K.) measuring to the nearest 10 g. Then the participants were seated in an upright position, which they held for the

3 – hour duration of the trial to avoid effects of postural changes on plasma volume (Hagan et al. 1978; Shirreffs & Maughan, 1994).

Ten minutes into the session, a canula was inserted in a superficial forearm vein, which allowed multiple blood samples to be drawn. The canula was kept patent between sample collections by flushing with heparinised sterile isotonic saline (10 units·ml⁻¹). Two resting (5 ml) blood samples were obtained ten minutes apart, and immediately after the second sample the participants ingested 400 ml of either still water (0.0 mosmol·l⁻¹), a glucose solution (1 g·kg⁻¹ body mass; 1039 ± 157 mosmol·kg⁻¹) or a glycerol solution (1 g·kg⁻¹ body mass; 2032 ± 309 mosmol·kg⁻¹). The subjects were allowed 3 min to ingest the drink. Blood samples (5 ml) were collected at 15 min interval for the first hour and then every 30 min for a further 90 – min period.

Sample analysis

Samples were analysed for haemoglobin concentration, haematocrit, blood glucose concentration, serum glycerol concentration and serum osmolality as described in the general methods chapter of this thesis. Haemoglobin concentrations and haematocrit were used to estimate changes in blood, red cell and plasma volumes as described by Dill and Costill (1974).

Statistical analysis

Two–factor repeated measures ANOVA was used to evaluate differences between trials. One–factor ANOVA was used to evaluate results over time when appropriate. Tukey post hoc pair wise comparisons were used to locate and isolate differences between trials and over time when appropriate.

Results

Baseline measurements were similar during the three different experimental trials (Table 3.1).

Table 3.1 Pre-trial body mass, haemoglobin concentration, haematocrit, blood glucose concentration, serum glycerol concentration and serum osmolality on all trials (Mean \pm SD); n = 9.

Measure	Water trial	Glucose trial	Glycerol trial	P value
Body mass (kg)	74.8 \pm 11.4	74.5 \pm 11.3	74.9 \pm 11.1	0.400
Hb (g·l ⁻¹)	152 \pm 10	151 \pm 12	151 \pm 7	0.662
Hct (%)	43.5 \pm 2.3	43.6 \pm 2.4	43.6 \pm 2.	0.943
Glucose (mmol·l ⁻¹)	5.35 \pm 0.30	5.37 \pm 0.36	5.53 \pm 0.29	0.451
Serum Glycerol (mmol·l ⁻¹)	0.04 \pm 0.01	0.04 \pm 0.01	0.04 \pm 0.01	0.776
Serum Osmolality (mosmol·kg ⁻¹)	291 \pm 6	293 \pm 5	291 \pm 5	0.540

Serum glycerol concentration (Table 3.2)

Two-way repeated measures ANOVA on serum glycerol showed a main effect of trial (P = 0.000), time (P = 0.000) and an interaction (P = 0.000). Serum glycerol increased following glycerol ingestion (P < 0.001) and remained elevated throughout the experimental trial. The value obtained at 30 min following glycerol ingestion was higher (P < 0.050) than the values obtained at all other time points in the glycerol trial, except values at 15 and 45 min.

Table 3.2 Serum glycerol concentration ($\text{mmol}\cdot\text{l}^{-1}$); $n = 9$. * Denotes difference between the glycerol trial and the other two trials ($P = 0.000$). † Denotes difference from baseline in the glycerol trial ($P = 0.000$).

Time (min)	0	15	30	45	60	90	120	150
Water	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
trial	± 0.01	± 0.01	± 0.01	± 0.01	± 0.01	± 0.01	± 0.01	± 0.01
Glucose	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
trial	± 0.02	± 0.02	± 0.01	± 0.02	± 0.02	± 0.02	± 0.02	± 0.02
Glycerol	0.04	10.38*†	12.66*†	10.90*†	8.83*†	6.88*†	6.16*†	5.67*†
trial	± 0.01	± 3.98	± 3.40	± 2.68	± 1.76	± 2.28	± 0.81	± 0.77

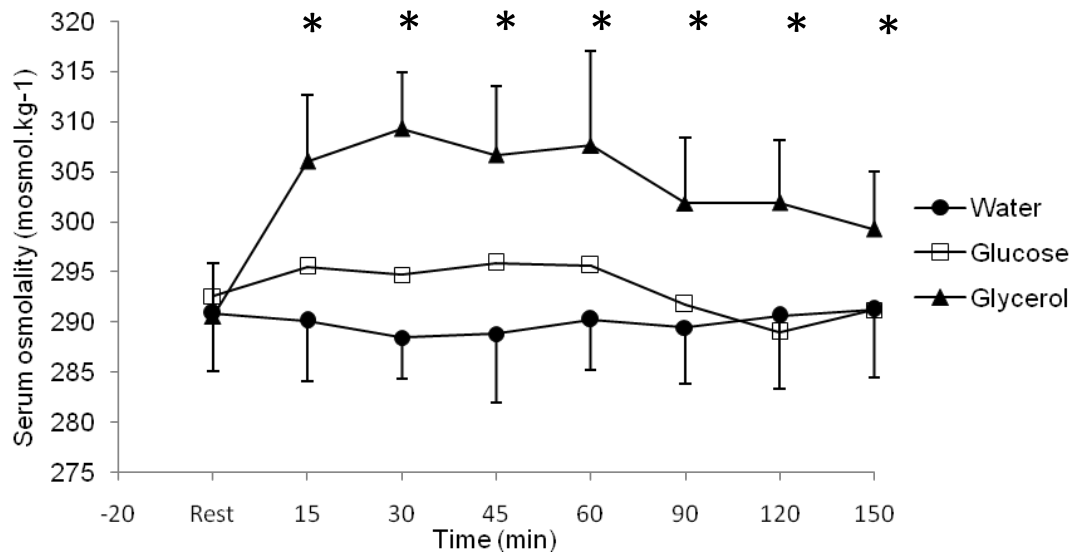
Serum osmolality (Fig. 3.1)

Resting serum osmolality (Table 3.1) was slightly over the anticipated values of $280 - 285 \text{ mosmol}\cdot\text{kg}^{-1}$ (Geigy Scientific Tables, 1962). This might be due to the fact that the subjects had fasted overnight before the main trials and they had not had any water or fluid intake during the fasting period.

Two-way repeated measures ANOVA on serum osmolality showed a main effect of trial ($P = 0.000$), time ($P = 0.000$) and an interaction ($P = 0.000$). One way ANOVA analysis over time in the glycerol trial showed that serum osmolality increased after glycerol ingestion to $306 \pm 7 \text{ mosmol}\cdot\text{kg}^{-1}$ at 15 min, $309 \pm 6 \text{ mosmol}\cdot\text{kg}^{-1}$ at the 30 min point, and remained at higher than baseline levels for 150 min ($P < 0.02$).

One way ANOVA analysis showed that serum osmolality did not change over time in the water trial ($P = 0.974$) nor in the glucose trial ($P = 0.165$) and remained at similar to base line (Water trial $291 \pm 6 \text{ mosmol}\cdot\text{kg}^{-1}$, glucose trial $293 \pm 5 \text{ mosmol}\cdot\text{kg}^{-1}$).

Figure 3.1 Serum osmolality ($\text{mosmol}\cdot\text{kg}^{-1}$). Points are means \pm SD; $n = 9$. * denotes glycerol time point significantly different ($P < 0.05$) from water point, glucose point and baseline.



Estimated changes in blood (Fig. 3.2), plasma (Fig. 3.3) and red cell volume (Fig. 3.4).

The increase in serum osmolality after glycerol ingestion was associated with an increase in blood volume and plasma volume.

Two-way repeated measures ANOVA on blood volume showed a main effect of trial ($P = 0.046$), time ($P = 0.000$) and an interaction ($P = 0.003$) (Figure 3.2). Post hoc analysis showed that blood volume following glycerol ingestion was greater than in the other two trials during the first 45 ($P < 0.003$). One way ANOVA over time for each trial showed that blood volume did not change over time in the water ($P = 0.072$) and glucose trials ($P = 0.694$). In the glycerol trial, blood volume had increased by 15 min post-ingestion ($P = 0.013$) and reached its highest mean value ($4.0 \pm 2.1\%$ increase) at 30 min ($P = 0.004$) after glycerol intake.

Two-way repeated measures ANOVA on red blood cells volume (RCV) showed that there was no trial effect ($P = 0.294$), no time effect ($P = 0.713$) and no interaction ($P = 0.326$) (Fig. 3.3).

Two-way repeated measures ANOVA on plasma volume showed a main effect of trial ($P = 0.026$), time ($P = 0.000$) and an interaction ($P = 0.000$) (Figure 3.4). Post-hoc analysis showed that plasma volume in the glycerol trial was significantly higher than in the other two trials for the first 45 min of the trial ($P = 0.000$). In the glycerol trial, plasma volume increased above baseline at 15 min after ingestion by $6.0 \pm 3.8\%$ ($P = 0.007$) and peaked at 30 min as it expanded by $7.2 \pm 4.0\%$ ($P = 0.001$). During this period of time, plasma volume in the other two trials did not change from baseline (water trial $P = 0.191$, glucose trial $P = 0.513$).

Figure 3.2 Blood volume changes (%). Points are means \pm SD; $n = 9$. * denotes glycerol time point significantly different ($P < 0.05$) from water and glucose trials at the same time point.

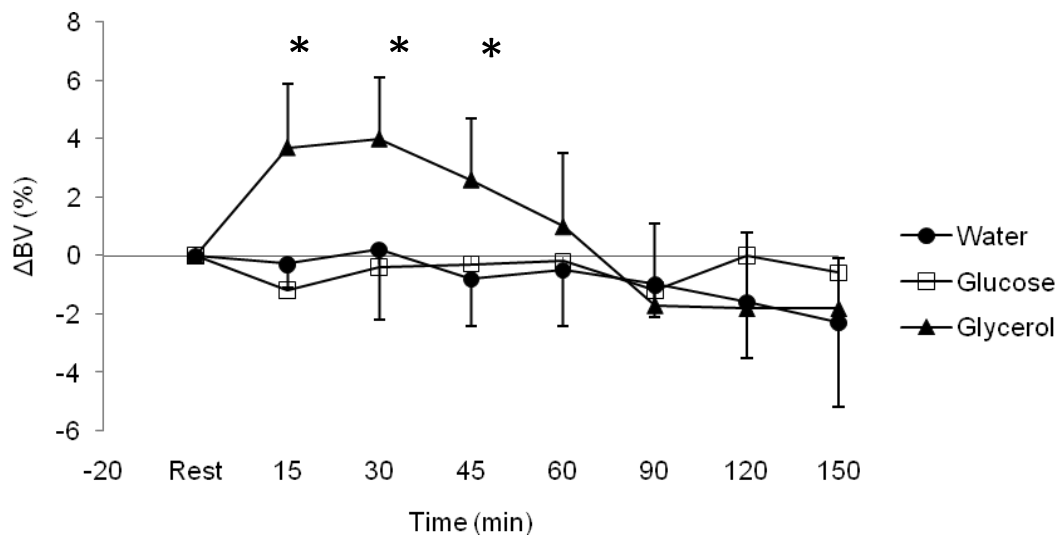


Figure 3.3 Red blood cell volume changes (%). Points are means \pm SD; n = 9.

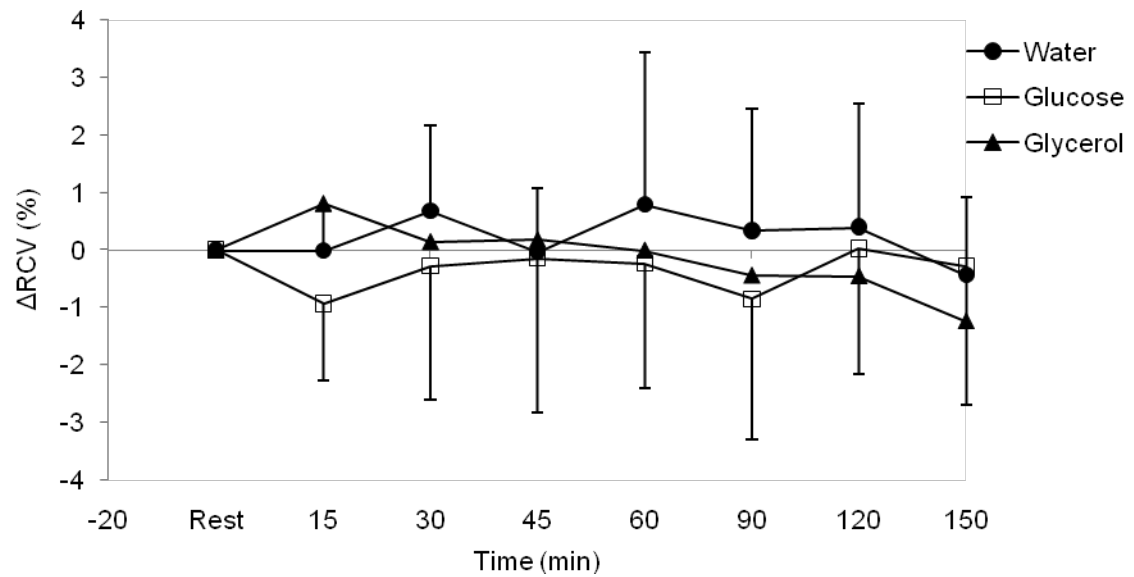
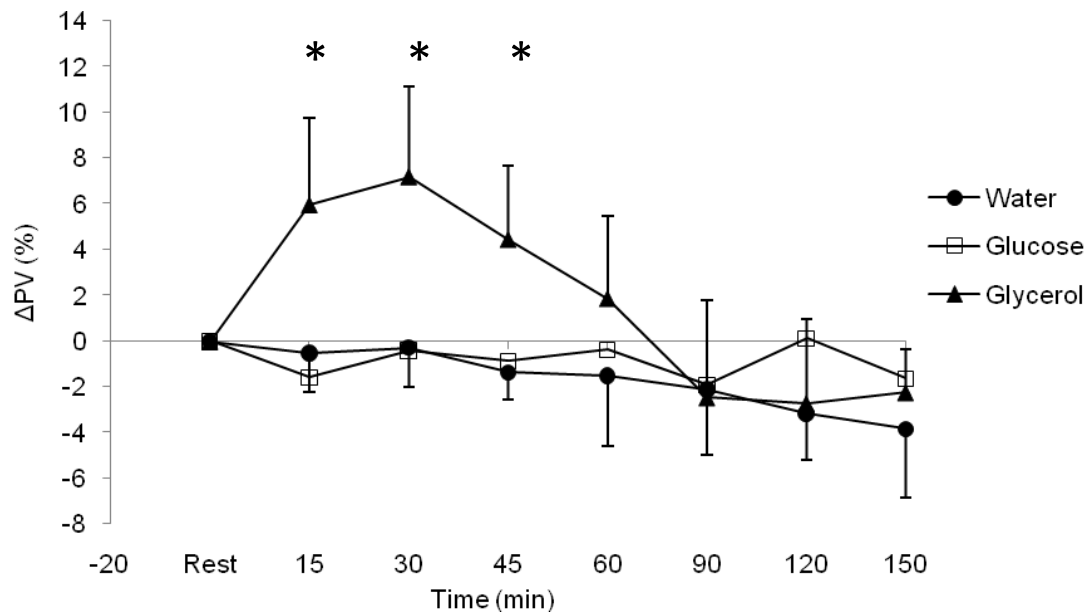


Figure 3.4 Plasma volume changes (%). Points are means \pm SD; n = 9. * denotes glycerol time point significantly different ($P < 0.050$) from water and glucose trials at the same time point.



Blood glucose concentration (Table 3.3)

Two-way repeated measures ANOVA on the blood glucose data showed a main effect of trial ($P = 0.000$), time ($P = 0.000$) and an interaction ($P = 0.000$).

One-way ANOVA over time showed that blood glucose increased above baseline only in the glucose trial ($P < 0.005$) to $8.57 \pm 1.57 \text{ mmol}\cdot\text{l}^{-1}$ at 30 min and remained elevated for 60 min following ingestion of the glucose solution.

In the water trial, blood glucose was $5.35 \pm 0.30 \text{ mmol}\cdot\text{l}^{-1}$ at baseline and it did not change over time ($P = 0.433$). Similarly, in the glycerol trial blood glucose was 5.53 ± 0.29 at baseline and did not change over time ($P = 0.641$).

Table 3.3 Blood glucose concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; $n = 9$. * Denotes difference from baseline within trial ($P < 0.003$). † Denotes difference between the glucose trial and the other two trials ($P < 0.050$)

Time (min)	0	15	30	45	60	90	120	150
Water trial	5.35 ± 0.30	5.08 ± 0.29	5.11 ± 0.40	5.12 ± 0.43	5.03 ± 0.43	5.05 ± 0.46	5.27 ± 0.51	5.39 ± 0.51
Glucose trial	5.37 ± 0.36	8.05*† ± 1.04	8.57*† ± 1.57	8.03*† ± 2.09	7.89*† ± 1.50	7.09† ± 1.18	6.76† ± 1.17	5.48 ± 0.97
Glycerol trial	5.53 ± 0.29	5.55 ± 0.43	5.67 ± 0.39	5.73 ± 0.47	5.78 ± 0.50	5.72 ± 0.49	5.83 ± 0.37	5.86 ± 0.36

Discussion

The purpose of this study was to look for any effects of water, glucose or glycerol ingestion on body water distribution. In the human body, cell membranes are highly permeable to water and the movement of water depends on osmotic gradients across the cell membrane (Lang et al., 1998). Water and glucose ingestion did not cause any changes in blood or plasma volume, whereas following glycerol ingestion blood and plasma volume increased for a period of 45 min.

Glucose ingestion did not produce any statistically significant changes in body water distribution. On this trial the glucose drink was hypertonic, containing an average of $187 \pm 28 \text{ g}\cdot\text{l}^{-1}$ glucose with an osmolality of $1039 \pm 157 \text{ mosmol}\cdot\text{kg}^{-1}$. A transient change in body water distribution could have been expected as a result of the ingested solution's high osmolality. Merson et al. (2002) reported decreases in blood and plasma volume 20 min following ingestion of a 12% glucose solution (compared with ingestion of a 2% glucose solution) due to a net flow of water into the intestine. Leiper and Maughan (1986) reported that perfusion of a hypertonic solution with an osmolality of $488 \pm 53 \text{ mosmol}\cdot\text{kg}^{-1}$ in the jejunum resulted in net water secretion into the small intestine. Evans et al. (2009) also reported a transient 3% decrease in plasma volume following a 10% glucose solution (osmolality $565 \text{ mosmol}\cdot\text{kg}^{-1}$). Contrary to the above findings, in the present study there were no significant changes either in blood or plasma volumes following glucose ingestion. The range of blood volume change at 30 min was between +2.7% and -5.6%. Plasma volume values ranged between +0.6% and -3.5% at 15 min, and at 30 min values ranged between +2.2% and -7.3%. It is clear that a large variation exists between subjects and this might have prevented the results from reaching statistical significance. This variation could be due to the difference in the rate of gastric emptying between subjects (Vist & Maughan, 1994).

Glycerol ingestion increased blood and plasma volume for a period of 45 min. Serum glycerol was $12.66 \pm 3.40 \text{ mmol}\cdot\text{l}^{-1}$ 30 min after ingestion and this was associated with an increase of serum osmolality of $\sim 15 \text{ mosmol}\cdot\text{kg}^{-1}$. At this time point blood volume increased by $4.0\% \pm 2.1$ and plasma volume increased by $7.2\% \pm 4.0$ and these increases were similar to those observed by Gleeson et al. (1986) who provided their subjects with a similar volume and amount of glycerol solution (400 ml solution, $1 \text{ g}\cdot\text{kg}^{-1}$ glycerol, $2100 \text{ mosmol}\cdot\text{kg}^{-1}$). Because glycerol rapidly equilibrates across the erythrocyte membrane (Roudier et al., 1998), red cell volume was not different amongst trials, meaning that increases in blood and plasma volume were solely due to a net increase of water in the plasma. This increase in the vascular space was attributed to the osmotic effect of the hypertonic glycerol solution (osmolality $2032 \pm 309 \text{ mosmol}\cdot\text{kg}^{-1}$).

The distribution of fluid between intracellular and extracellular compartments is determined mainly by the osmotic gradient across the cell membrane (Guyton & Hall, 1996; Maughan, 1998). Gleeson et al (1986) showed that ingestion of glycerol results in a marked rise in the extracellular glycerol concentration and osmolality, two facts that were also observed in the present study. This could favour a water shift from the intracellular space into the extracellular space, resulting in cellular dehydration.

In normal conditions there is an osmotic equilibrium between intracellular and extracellular fluids and glycerol is believed to be distributed evenly in all water compartments (Robergs and Griffin, 1998) except the cerebral spinal fluid due to the influences of the brain blood barrier (Nelson & Robergs, 2007). In the present study, if indeed, glycerol was equally distributed in all water compartments, then the increase in the vascular space would reflect an increase in total body water. While body water was not measured directly, only relative changes of blood, plasma and red blood cells volume over time were estimated using the Dill and Costill (1974) equations, but clearly insufficient water was ingested with the glycerol solution to allow total body water to increase to the same extent as did plasma volume. Plasma volume is estimated to be about 4.3% of body mass (or

3200 ml in the present study) as calculated from data obtained in studies where plasma volume was directly measured with the Evans blue dye technique (Coyle et al, 1986; Coyle et al, 1990; Green et al, 1987). On the glycerol trial in the present study, plasma volume increased by 7.2% and this corresponds to an additional ~216 ml. As plasma volume is considered to be approximately 1/5th of extracellular water (Guyton & Hall, 1996) and calculations from data obtained from Battistini et al. (1994), Coyle et al. (1986, 1990), Ellis (2000), Green et al. (1987), Ritz and investigators (2000) (where blood volume, total body water, extracellular and intracellular water volumes were measured with tracer techniques), extracellular water on the glycerol trial would have to have increased by about 1080 ml to match the plasma volume expansion seen in the present study. Extracellular water (measured with bromide dilution) has been reported to be approximately 35% of total body water (measured with deuterium oxide technique) in recreationally active people (Battistini et al, 1994). This would have meant that, on the glycerol trial, total body water would have to have increased by ~3085 ml if glycerol were readily distributed equally in all body water compartments. Using data on body water from a review paper by Ellis (2000) where extracellular water was considered to be 39% of total body water, total body water on the glycerol trial in the present study should have increase by ~2769 ml. However, the total volume of the ingested glycerol/water solution in the present study was only 400 ml and no other fluids were ingested on this trial. Therefore it is likely that glycerol ingestion led to a transient altered body water distribution, with the amount of intravascular expansion suggesting that this space was the main beneficiary of the osmotic water flux possibly at the expense of intracellular water. For any osmotic effect to occur at the cell membrane, a difference in glycerol concentration would be required between the intracellular and extracellular space. Following ingestion, glycerol is absorbed in the small intestine and to a smaller degree in the stomach (Nelson & Robergs, 2007). Although it is well accepted that glycerol absorption happens though passive diffusion (Lin, 1977) recent data has shown that its transport across the intestinal wall is carrier-mediated and saturable (Kato et al., 2005). The absorption rate of ingested glycerol has been reported to

vary between 1 and 1.4 g·min⁻¹ (Massicotte et al., 2006), and up to 3 g·min⁻¹ (Gleeson et al., 1986), and individual differences may influence peak serum glycerol concentration (Nelson and Robergs, 2007). Provided that glycerol enters both extracellular (vascular and interstitial space) and intracellular water spaces, glycerol space had originally been reported to be 650 ml·kg⁻¹ body mass (Wilmore & Costill, 1974), but recent data from studies, where tracer techniques were used, has reported glycerol space to be as low as 270 ml·kg⁻¹ (Casazza et al., 2004; Massicotte et al., 2006). Therefore it is difficult to estimate the exact kinetics of the ingested glycerol in the present study. What is important regarding possible alterations in body water distribution is the glycerol concentration gradients between the vascular and non-vascular space, and the intracellular and extracellular space and glycerol cell permeability. In the present study, it appears that there was a net accumulation of glycerol in the vascular compared with the non-vascular space over the initial 45-to-60 min after ingestion. Thereafter, glycerol efflux from the circulation exceeded influx and concentration gradients, at least in the extracellular space appear to have equilibrated. In the present study, red blood cells volume did not appear to change on the glycerol trial. This was due to the fact that human red blood cells are highly permeable to glycerol (Carlsen & Wieth, 1976; Levitt & Mlekoday, 1983). Upon exposure of red blood cells to a hypertonic environment they quickly shrink; but then as glycerol enters through the aquaporine channel AQP3, which is highly permeable to glycerol, the cells regain their volume rapidly because of the accompanying water influx (Roudier et al., 1998). Overall, red blood cells have adequate osmoregulation mechanisms that make them less susceptible to osmotic stress. Red blood cells in vivo have been shown to maintain their volume in conditions of elevated plasma osmolality, which can occur following ingestion of a hypertonic solution (Evans et al., 2009; Gleeson et al., 1986) and during maximal exercise (van Beaumont et al., 1973). This is achieved via retention of their potassium content and intracellular water (Kilburn, 1965) and increase in erythrocyte-cytosol glycerol concentration. Red cell volume has been shown to remain stable even when plasma osmolality

rose to $326 \text{ mosmol}\cdot\text{kg}^{-1}$ during dynamic exhaustive forearm exercise (Maassen et al., 1998).

On the other hand, muscle cells have been shown to behave like a passive osmometer and decrease in volume when exposed to a hypertonic environment (Blinks, 1965; Usher-Smith et al., 2009). Although glycerol does permeate the sarcoplasmic membrane (Bozler, 1961; Howell, 1969), Krolenko (1969) showed that single muscle fibres shrink when they are exposed to solutions made hypertonic by addition of different penetrating substrates, including glycerol. Glycerol entry into the muscle cells, compared with erythrocytes, does not seem to happen at a very fast rate, and muscle cells initially shrink upon exposure to a hyper-osmotic glycerol solution, before regaining their initial volume when glycerol concentration equilibrates between the intracellular and extracellular space (Dulhunty et al., 1973). Caputo (1968) exposed single muscle fibres to 230 mM glycerol Ringer solution and the fibres lost 30% of their volume. Miyamoto and Hubbard (1972) exposed whole rat sartorii muscles to 420 mM glycerol Ringer solution and observed that the muscles lost 20% of their volume by 30 min, before starting to regain their volume. Glycerol and cell volume equilibrium time was 160 min, similar to 150 min found by Bozler (1961). Guo and Jensen (1999) infused intravenously $[\text{U-}^{13}\text{C}]$ glycerol into rats to evaluate whether glycerol equilibrates between plasma and intramuscular compartments. Their results showed that by the end of 1 hour of trace infusion, free glycerol of quadriceps, gastrocnemius and soleus muscles were only 40 – 70% of that of plasma glycerol. By the end of 2 hours of infusion, glycerol equilibrated in quadriceps and gastrocnemius, and glycerol equilibrated in the soleus muscle after 3 hours of infusion. On the glycerol trial in the present study, changes in vascular volume only were estimated. Blood and plasma volume returned to baseline by 60 min following glycerol ingestion, possibly due to entry of glycerol into the intracellular space, driving water away from the vascular space. This time frame compares to the findings of Terblanche et al. (1981) who reported that glycerol can take 1 – 2 hours after ingestion to be evenly distributed between the liver, blood and skeletal muscles.

What remains to be answered is whether the increase in plasma water was at the expense of intracellular or interstitial fluid. Hamilton et al. (1993) investigated fluid redistribution in isolated perfused cat calf muscle caused by rapid increases in plasma osmolality ($400 \text{ mosmol}\cdot\text{kg}^{-1}$ by adding NaCl). Their results showed that total tissue water decreased by more than 15% in less than 20 min, and that the changes in cell volume were similar to the changes in total tissue water. Interstitial fluid volume was not noticeably altered by osmolality. The authors concluded that the increase in plasma volume induced by hypertonic fluids may come entirely at the expense of intracellular water and not interstitial fluid. Based on the above, it is likely that muscle hydration on the glycerol trial in the present study had been negatively affected.

A decrease in cell hydration could affect cell metabolism, according to the cell volume theory (Haussinger, et al., 1994). In vivo, Keller et al., (2003) investigated the effects of hyper-osmolality on glucose kinetics in humans and reported an increased rate of endogenous glucose appearance in the plasma leading to an increased glucose concentration, due to hepatic glucose production in hyper-osmotic conditions ($296 \text{ mosmol}\cdot\text{kg}^{-1}$ following infusion of hypertonic NaCl). In the present study, on the glycerol trial, blood glucose concentration remained the same despite an increase in blood volume. This suggests that the total amount of glucose in the blood might have increased on this trial, implying increased glycogenolysis, which has been shown to occur when cells are exposed to a hypertonic environment (Lang et al, 1989). On the other hand, the maintenance of blood glucose concentration on the glycerol trial could well be attributed to the tight regulation of blood glucose concentration as glucose is the fuel for the central nervous system (Mougios, 2006; Wasserman, 1995) and a decrease in blood glucose would compromise glucose delivery to the brain.

Antolic et al. (2007) examined the in vitro effects of extracellular osmolality on cell volume and resting metabolism in mammalian skeletal muscle. Whole rat muscles were bathed in isosmotic ($290 \text{ mosmol}\cdot\text{kg}^{-1}$), hypo-osmotic ($190 \text{ mosmol}\cdot\text{kg}^{-1}$) and hyper-osmotic ($400 \text{ mosmol}\cdot\text{kg}^{-1}$) environments. Their results showed that extracellular hyper-osmolality resulted in decreases in muscle water content and

alterations in resting muscle metabolism, with lower ATP and PCr content. Judelson et al. (2008) studied the effect of hydration state on the endocrine and metabolic responses to resistance exercise. Hypohydration (by 2.5% and 5% body mass) increased circulating stress hormones (cortisol, epinephrine, norepinephrine) and lowered the resistance exercise-induced increases in circulating concentrations of testosterone. Based on these findings, the authors concluded that hypohydration can increase catabolism and potentially decrease anabolism, and that body water status is an important consideration in modulating the hormonal and metabolic responses to resistance exercise.

Glycerol solutions with high concentration, as in the present study, have been known to be a potent dehydrating agent (Frank et al., 1981) and high elevation of extracellular osmolality following glycerol feeding may well result in cell dehydration and tissue shrinkage (Gleeson et al., 1986; Maughan, 1998; Nelson & Robergs 2007).

Conclusion and future direction

Glycerol ingestion (400 ml solution, $1\text{g}\cdot\text{kg}^{-1}$ glycerol) resulted in high extracellular osmolality and a transient change in body water distribution. Plasma volume increased for a period of 45 min suggesting a shift of water into the extracellular space. This implies that cell hydration, including muscle cells, may have been compromised. Exposure to a hypertonic environment may possibly lead to cellular dehydration, which may have negative effects during or following exercise; increased glycolysis and glycogenolysis that may compromise the muscles' energy stores; and increased proteolysis and inhibition of insulin action that may restrict muscle growth. Although intracellular water cannot be quantified from the data obtained, it would be of interest to investigate whether there is any effect of high extracellular osmolality and the resulting changes in body water distribution on muscle function and exercise performance.

Chapter 4

Acute effects of water and glycerol ingestion on
body water distribution, during and after isometric
exercise at high intensity

Introduction

The effects of dehydration (as expressed by decreased total body water) on the cardiovascular system have been well established, but research on the effects of dehydration on strength has produced equivocal findings. In previous studies, dehydration resulted in either no changes in strength (Greiwe et al., 1998; Montain et al., 1998) or a decrease (Viitasalo et al., 1987; Bosco et al., 1974; Schoffstall et al., 2001). The method used to induce dehydration in the various studies could explain this discrepancy. In general, energy restriction, heat exposure and exercise in the heat used to induce dehydration all appeared to negatively affect muscle function (Greiwe et al., 1998, Judelson et al., 2007a). Therefore the outcome of the various strength tests could not solely be attributed to dehydration. Overall, hypohydration of 3 – 4% body mass reduces muscular strength by approximately 2% (Judelson et al., 2007a) and hypohydration has been shown to affect even brief bouts of isometric exercise (Torrainin et al., 1979).

Altered body water distribution may also influence cell volume and cellular metabolism (Ritz et al., 2003), without any reduction in total body water. Muscle tissue is approximately 75% water and adequate hydration is essential for normal cellular function (Jones et al., 2008). Cell hydration and cell volume seems to be a signalling key for muscle cell metabolism and function (Haussinger et al., 1994). Decreased cellular hydration might trigger a catabolic pattern of cellular function (Haussinger & Lang, 1991), disturb muscle metabolism and may potentially affect muscle function and exercise performance.

Although changes in total body hydration are easily monitored, alterations in cellular hydration cannot be easily determined in a total body situation, especially when individuals are exercising. Invasive methods (insertion of catheters and muscle biopsies) have been used to describe changes in muscle water and ion shifts during dynamic exercise (Sjogaard & Saltin, 1982; Sjogaard et al., 1985), but the effects of cellular hydration during exercise have not been fully investigated, and almost all the information regarding the effects of cellular hydration and

volume on cell metabolism and function comes from in vitro studies (Lang et al., 1998; Low et al., 1996).

In an exercise setting, in order to address the issue of altered body water distribution and altered cellular hydration, glycerol ingestion may prove to be a useful scientific tool. Changing body water distribution (without causing dramatic changes in total body water) can be achieved by the ingestion of a concentrated glycerol solution. This has been shown to alter body water distribution by increasing extracellular body water as expressed by changes in blood volume and plasma volume, for a period of 45 min (detailed description in chapter 3 of this thesis). When glycerol is given at $1 \text{ g}\cdot\text{kg}^{-1}$ body mass, diluted in a small volume of water, serum osmolality increases by $\sim 15 \text{ mosmol}\cdot\text{kg}^{-1}$, resulting in a water shift into the extracellular space, indicating a reduction in intracellular hydration (Guyton & Hall, 1996; Robergs & Griffin, 1998).

Glycerol is not a major substrate for energy production during exercise. Glycerol can be metabolised to glucose through gluconeogenesis (Nelson & Robergs, 2007), but humans cannot utilize glycerol as a gluconeogenic substrate rapidly enough to serve as a fuel during strenuous exercise (Gleeson et al, 1986). Therefore, it can be used solely for the purpose of altering serum osmolality and possibly body water distribution.

The aim of this study was to investigate acute effects of glycerol ingestion and changes in body water distribution on muscular function during isometric exercise at high intensity.

Materials and Methods

Following ethical approval by the Loughborough University Ethical Advisory Committee, ten healthy male volunteers participated in this study. After a written and oral explanation of all procedures and their right to withdraw at any point

without obligation, each subject signed a written consent form. All participants were physically active and accustomed to resistance exercise. Their age, height and body mass were, respectively, (mean \pm SD) 25 ± 3 y, 178 ± 8 cm, and 78.0 ± 9.2 kg. Each subject participated in two experimental trials in randomised order, which was assigned using a Latin square design.

Experimental procedure

Each participant visited the laboratory on three occasions, separated by at least 7 days. The first visit was used to measure maximum voluntary contraction (MVC) of the quadriceps muscles and to familiarise each participant with the exercise protocol on the isometric chair. The second and third visits were when the two main experimental trials occurred.

Isometric exercise set-up

Each participant was seated and strapped into an isometric chair according to Maughan et al. (1983) and as described in detail in the methods chapter of this thesis. Hip and knee joints were flexed at $\sim 90^\circ$. An inelastic cable was attached to the exercising leg, just above the ankle joint on one end, and to a strain gauge on the other end. As each participant tried to extend the knee, the force generated was measured by the strain gauge. The analogue signal from the gauge was amplified, digitised and transmitted to a computer, where a digitised graph of the produced force was displayed on a monitor in real time, so each participant had visual feedback of the force he was generating.

First laboratory visit – MVC measurement and exercise protocol familiarisation

MVC measurement: MVC was measured for both legs. The participants performed single leg maximal quadriceps muscle activations of 3 s with 1 min recovery in between. All activations were executed on the same leg before switching to the other leg. The stronger leg was chosen as the exercising leg for the main trials.

After the MVC test, there was a familiarisation session with the exercise protocol that was to be used in the main trials. Participants performed six efforts of 6 s at 90% MVC with 1 min rest, using their stronger leg.

Main trials

The two main trials were separated by at least 7 days and not more than 15 days, starting at the same time in the morning. Participants were asked to fast overnight, and to follow a similar activity and food intake pattern for the 24 hours preceding each trial. Participants assured us that they had adhered to these instructions.

On arrival at the laboratory, each participant voided and then assumed an upright sitting position. Their sitting position was similar to the sitting position on the isometric chair in order to control for any plasma volume changes due to posture. One arm was submerged into hot water (42°C) for 10 min. Then an indwelling canula was inserted into a forearm vein, and stayed in place throughout the session to allow us to draw multiple blood samples.

After a 30 min resting period, each participant ingested either water (6 ml·kg⁻¹ body mass) or a glycerol solution (1 g·kg⁻¹body mass, 6 ml·kg⁻¹body mass). The average glycerol drink contained 78 ± 9 g glycerol and had a volume of 468 ± 53 ml. Each participant was allowed 3 min to fully ingest the drinks. Following ingestion, the participant remained seated for a further 25 min period before moving to the isometric chair.

Exercise started 30 min after ingestion. The participant performed 12 quadriceps activations of his stronger leg at 90% MVC intensity with 1 min rest between efforts.

Following completion of the exercise protocol, the participant remained seated in the laboratory for 90 min in order to monitor body water changes as indicated by blood and plasma volume changes (Dill and Costill, 1974) during the recovery period. Because there were no visits to the toilet during the trials, there were no

changes in total body water due to urination, or changes in blood volume due to unnecessary postural changes as all subjects adhered to the trial protocol.

Blood sampling

During the initial resting period, blood samples were taken 20 and 10 min before ingestion and 25 min after ingestion (before - exercise point). During exercise, blood was drawn immediately after the 4th, 8th and 12th effort. During the recovery period, blood was taken at 4, 15, 30, 45, 60 and 90 min.

Sample analysis

Blood samples were analysed for haemoglobin concentration, haematocrit, blood glucose and blood lactate. Haemoglobin concentrations and haematocrit were used to estimate changes in blood, red cell and plasma volumes as described by Dill and Costill (1974).

Serum samples were analysed for osmolality and glycerol.

All analyses were performed as described in the general methods section of this thesis.

Statistical Analysis

For data that were normally distributed, a two-way ANOVA was used to find differences among trials. Where appropriate, a Tukey post hoc test was used to distinguish differences among trials. A one-way ANOVA was also used to find differences over time during a trial.

Data that were not normally distributed were analyzed with non-parametric statistical tests.

All data are presented as mean \pm standard deviation of the mean (SD), and the median and range for values for each time point in the trials. For all statistical analyses, significance was set at $P < 0.050$.

Results

Baseline measurements were similar during the two main experimental trials (Table 4.1).

Table 4.1 Pre – trial body mass, Hb concentration, Hct, blood glucose concentration, serum glycerol concentration, serum osmolality and blood lactate (Mean \pm SD); n = 10.

Measure	Water trial	Glycerol trial	P value
Body mass (kg)	77.87 \pm 9.01	77.85 \pm 9.30	0.934
Hb (g·l ⁻¹)	149 \pm 1	147 \pm 1	0.541
Hct (%)	43.7 \pm 3.4	42.9 \pm 1.9	0.553
Blood glucose (mmol·l ⁻¹)	5.27 \pm 0.33	5.34 \pm 0.32	0.298
Serum glycerol (mmol·l ⁻¹)	0.03 \pm 0.01	0.03 \pm 0.01	0.167
Serum osmolality (mosm·kg ⁻¹)	282 \pm 5	284 \pm 4	0.113
Blood lactate (mmol·l ⁻¹)	0.61 \pm 0.26	0.69 \pm 0.28	0.419

Serum glycerol concentration (Fig. 4.1)

Two-factor repeated measures ANOVA on serum glycerol concentrations showed a main effect of trial (P = 0.000), time (P = 0.000) and an interaction (P = 0.000).

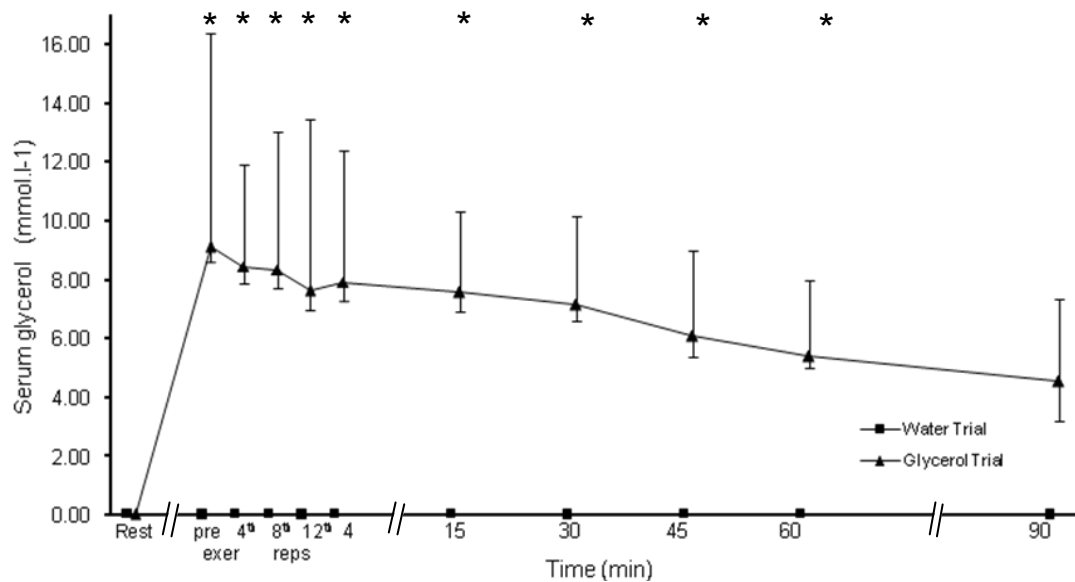
One-way ANOVA analysis over time showed that serum glycerol increased following glycerol ingestion to 10.26 \pm 3.79 mmol·l⁻¹ (median 9.11 mmol·l⁻¹; range 4.55 – 16.36 mmol·l⁻¹) before exercise. Serum glycerol remained elevated throughout the glycerol trial. At the end of exercise, serum glycerol was 8.28 \pm 2.86 mmol·l⁻¹ (median 7.64 mmol·l⁻¹; range 4.14 – 13.48 mmol·l⁻¹), and at 90 min after exercise, it was 4.87 \pm 1.48 mmol·l⁻¹ (median 4.87 mmol·l⁻¹; range 3.16 –

7.35 mmol·l⁻¹), which were significantly higher than the resting serum glycerol value of 0.03 ± 0.01 mmol·l⁻¹.

During the water trial, serum glycerol did not change over time (P = 0.775) and remained at baseline 0.03 ± 0.01 mmol·l⁻¹.

Figure 4.1 Serum glycerol concentration (mmol·l⁻¹). Points denote median (min,max); n = 10.

* Denotes difference between trials (P < 0.05).



Serum Osmolality (Fig. 4.2)

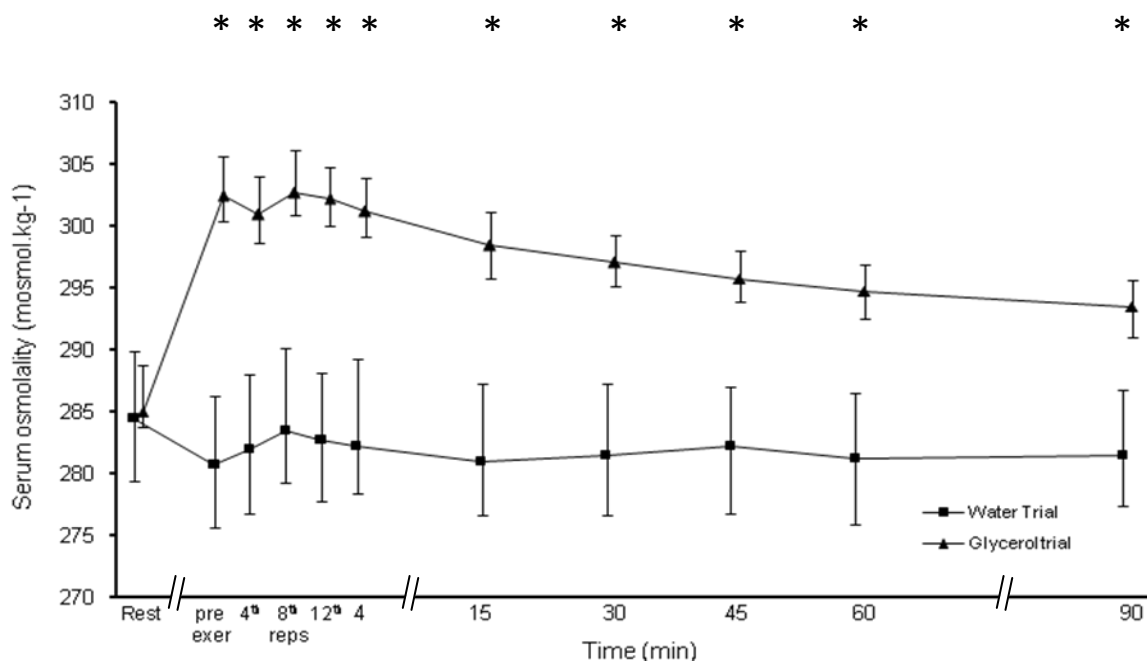
Two-way repeated measures ANOVA on serum osmolality showed a main effect of trial (P = 0.000), time (P = 0.000) and an interaction (P = 0.000).

One-way ANOVA analysis over time for the glycerol trial showed that serum osmolality increased following glycerol ingestion and reached 300 ± 8 mosmol·kg⁻¹ (median 303 mosmol·kg⁻¹; range 287 – 307 mosmol·kg⁻¹) before exercise started. Serum osmolality remained elevated throughout the experimental trial. At 90 min after exercise, serum osmolality was 293 ± 4 mosmol·kg⁻¹ (median 294 mosmol·kg⁻¹; range 286 – 300 mosmol·kg⁻¹), which was significantly higher than the resting value.

In the water trial, serum osmolality did not change over time ($P = 0.926$) and remained close to resting levels ($282 \pm 5 \text{ mosmol}\cdot\text{kg}^{-1}$; median $285 \text{ mosmol}\cdot\text{kg}^{-1}$; range $275 - 287 \text{ mosmol}\cdot\text{kg}^{-1}$).

The changes in serum osmolality were greater than the changes expected ($\sim 10 \text{ mosmol}\cdot\text{kg}^{-1}$) based on the increase in serum glycerol. It is important to note though that the increases in blood lactate and any changes in serum electrolytes (not measured in the present study) could account for the rise in serum osmolality.

Figure 4.2 Serum Osmolality ($\text{mosmol}\cdot\text{kg}^{-1}$). Points are median (min,max); $n = 10$. * denotes glycerol time point significantly different ($P < 0.05$) from water point.



Estimated changes in blood volume, red cell volume and plasma volume

Blood volume changes (Fig. 4.3)

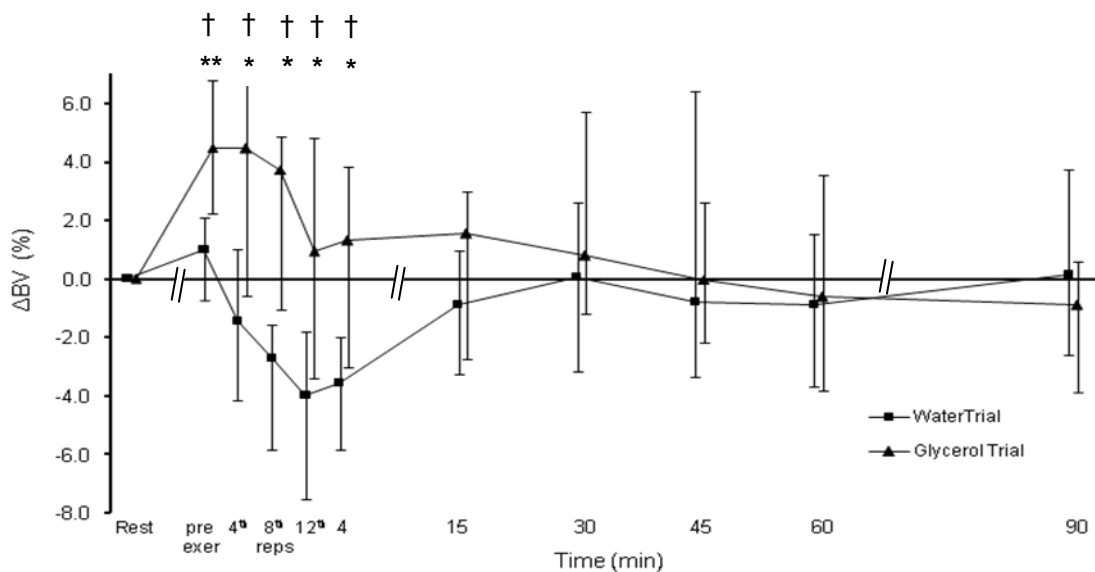
Two-way repeated measures ANOVA on blood volume showed a main effect of trial ($P = 0.004$), time ($P = 0.005$) and an interaction ($P = 0.001$).

One-way ANOVA analysis over time showed that blood volume increased following glycerol ingestion ($P = 0.009$) by $4.5 \pm 1.7\%$ (median 4.5%; range 2.6 – 6.3%). Blood volume did not change following water ingestion ($P = 0.994$). During exercise, blood volume decreased in both trials in relation to pre-exercise values. On the water trial, blood volume decreased by $5.2 \pm 2.4\%$ ($P = 0.000$) and on the glycerol trial, blood volume decreased by $3.9 \pm 4.4\%$ ($P = 0.000$) by the end of exercise. Blood volume at 4 min after exercise was still lower than the pre-exercise point in both trials (water trial - $4.4 \pm 1.8\%$, $P = 0.000$; glycerol trial - $4.2 \pm 4.0\%$, $P = 0.000$).

On the water trial, blood volume returned to the pre-exercise resting baseline at 15 min following exercise ($P = 0.080$), and it remained at resting levels for the remainder of the study.

As expected on the glycerol trial, blood volume did not return to the pre-exercise value ($P < 0.050$), instead it remained at the initial resting pre-glycerol ingestion value ($P = 1.000$ at end of exercise).

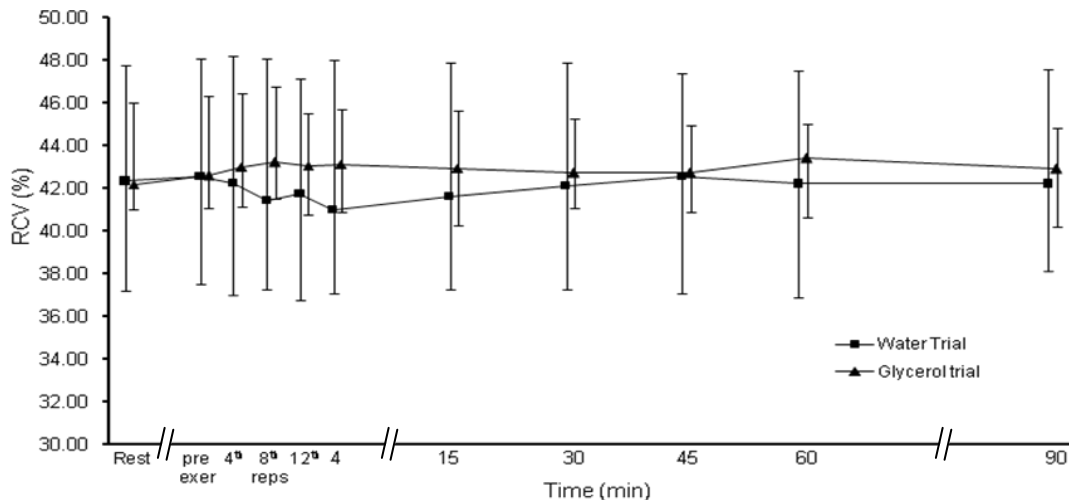
Figure 4.3 Blood volume changes (%). Points are median (min,max); $n = 10$. † Denotes difference between trials ($P < 0.050$). * Denotes difference from pre-exercise point in both trials ($P < 0.050$). ** Glycerol pre-exercise point is higher than glycerol rest point ($P = 0.009$).



Red cell volume (Fig. 4.4)

Two-way repeated measures ANOVA on red cells volume showed that there was no trial effect ($P = 0.867$), no time effect ($P = 0.103$) and no interaction ($P = 0.188$).

Figure 4.4 Estimated percentage red blood cells volume. Points are median (min,max); $n = 10$.



Plasma volume changes (Fig. 4.5)

Two-way repeated measures ANOVA on plasma volume showed a main effect of trial ($P = 0.000$), time ($P = 0.006$) and an interaction ($P = 0.001$). One-way ANOVA analysis over time within trials showed that plasma volume increased following glycerol ingestion ($P = 0.000$) by $7.8 \pm 2.0\%$ (median 7.5%; range 4.0 – 9.9%).

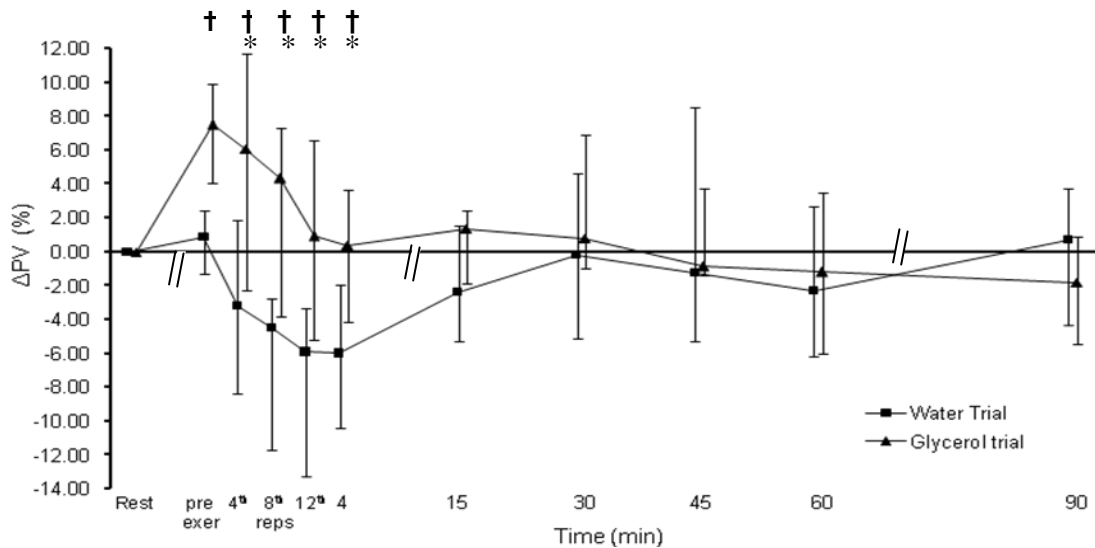
There was no change in plasma volume following water ingestion ($P = 0.507$).

During exercise, plasma volume decreased from the pre-exercise value similarly in both trials. On the water trial, plasma volume decreased by $7.4 \pm 3.4\%$ by the end of exercise ($P = 0.001$), and on the glycerol trial, plasma volume decreased by $7.5 \pm 5.7\%$ ($P = 0.025$). During exercise, plasma volume in the glycerol trial was higher than that in the water trial ($P < 0.050$) because of the higher value at the beginning of exercise.

At 4 min following exercise, plasma volume was still lower than the pre-exercise value on both trials (water trial by $-6.6 \pm 2.9\%$, $P = 0.002$; glycerol trial by $-8.2 \pm$

4.4%, $P = 0.003$). At 15 min after exercise, plasma volume returned to the pre-exercise resting level in the water trial ($P = 0.912$). As expected on the glycerol trial, plasma volume did not return to the pre-exercise value, but stayed at the pre-glycerol ingestion values ($P = 0.900$).

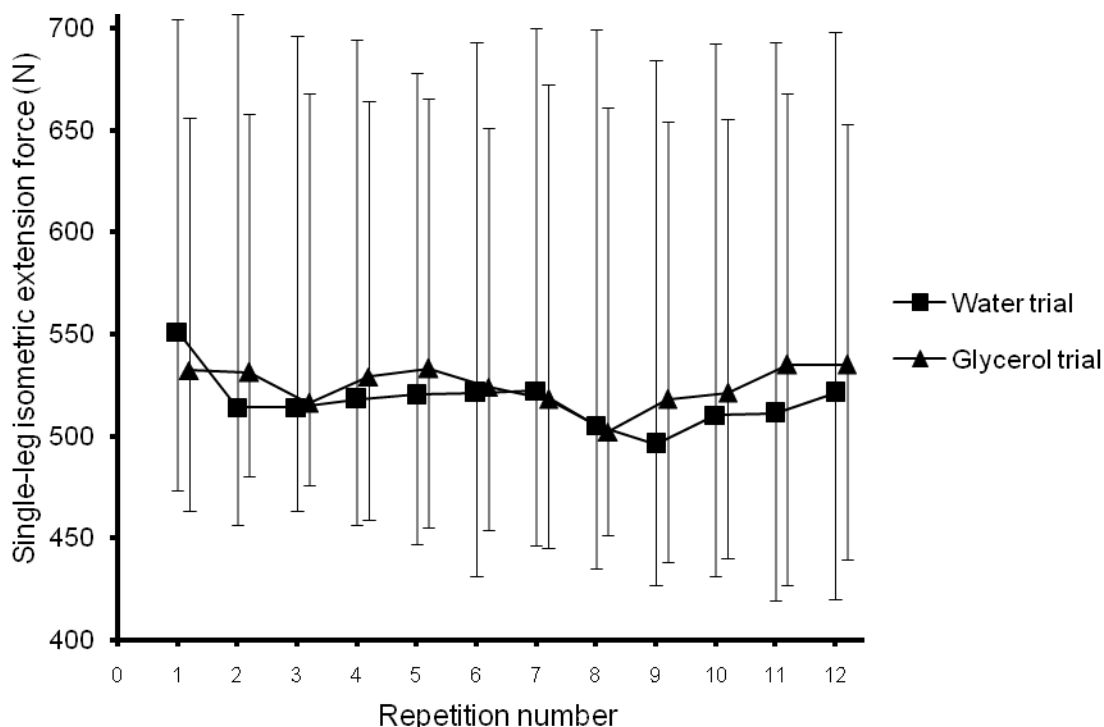
Figure 4.5 Plasma volume changes (%). Points are median (min,max); $n = 10$. † denotes glycerol time point significantly different ($P < 0.050$) from water point. * denotes time point significantly different to pre-exercise point ($P < 0.050$) in both conditions.



Isometric exercise results (Fig. 4.6)

The data obtained for single-leg isometric extension force were not normally distributed and were analysed with non-parametric statistical tests. A Friedman test showed that there was no effect of trial ($P = 0.960$), no effect of time ($P = 0.930$), and no interaction ($P = 0.100$).

Figure 4.6 Isometric force production (N). Bars are median (min,max); n = 10.



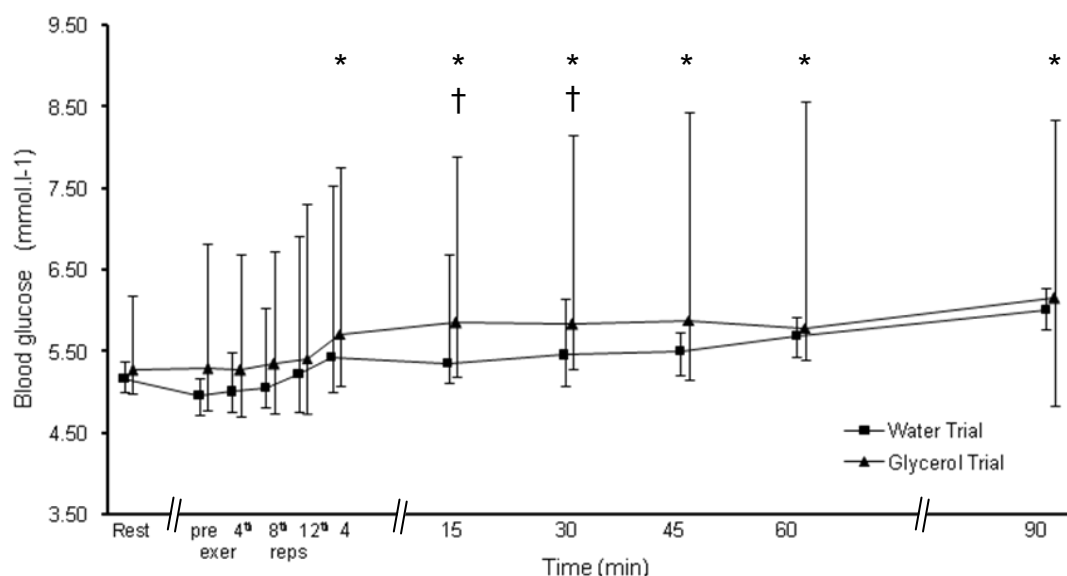
Blood glucose concentration (Fig. 4.7)

The data obtained for blood glucose were not normally distributed and were analysed with non-parametric statistical tests. A Friedman test showed that there was an effect of trial ($P = 0.001$) and time ($P = 0.001$), but no interaction ($P = 0.0995$).

A Mann-Whitney test for the glycerol trial revealed that blood glucose increased slightly at 4 min of recovery ($P = 0.031$) and remained higher than baseline throughout the recovery period ($P \leq 0.026$). There was no blood glucose change over time on the water trial.

Comparisons between trials showed that blood glucose on the glycerol trial was higher than the water trial at 15 min ($P = 0.034$) and 30 min ($P = 0.038$) in the recovery period.

Figure 4.7 Blood glucose concentration ($\text{mmol}\cdot\text{l}^{-1}$). Points are means \pm SD; $n = 10$. * Denotes time point significantly different from rest point in the glycerol trial. † Denotes time point in the glycerol trial significantly different from time point in the water trial.



Blood lactate concentration (Fig. 4.8)

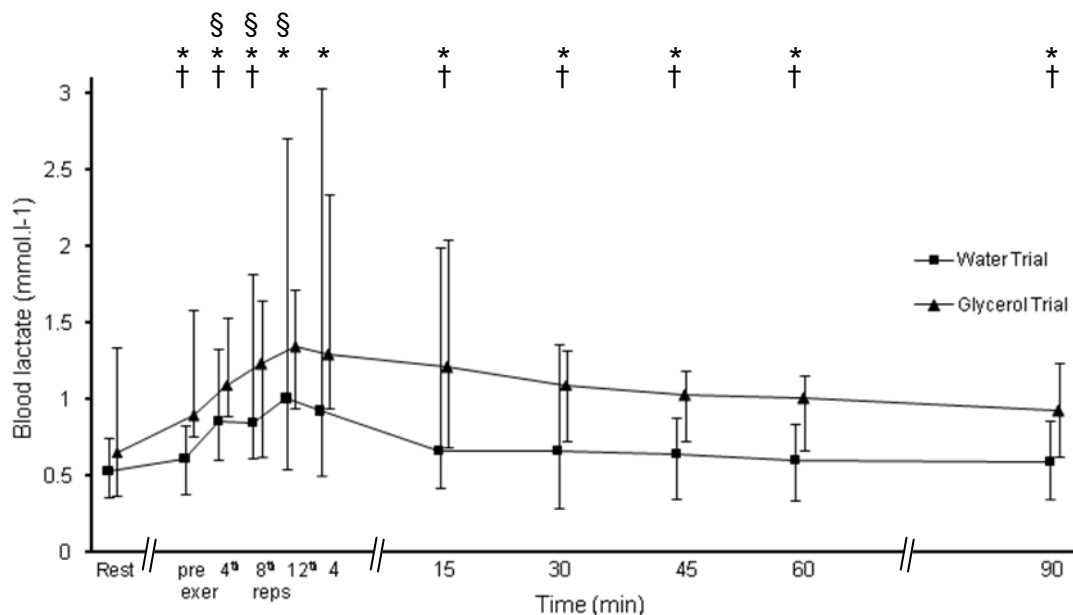
The data obtained for blood lactate were not normally distributed and were analysed with non-parametric statistical tests.

A Friedman test showed that there was an effect of trial ($P = 0.001$), time ($P = 0.001$), but no interaction ($P = 0.985$).

A Mann-Whitney test for the glycerol trial revealed that blood lactate increased at the pre-exercise point ($P = 0.011$) following glycerol ingestion and remained higher than baseline throughout the trial ($P < 0.050$). On the water trial, blood lactate increased above baseline during exercise (repetitions 4, 8 and 12; $P = 0.001$).

Comparisons between trials (Mann-Whitney test) revealed that blood lactate on the glycerol trial was higher than on the water trial at the pre-exercise point following glycerol ingestion ($P < 0.001$) and remained higher almost throughout the duration of the trials ($P < 0.030$) except at repetition 12 ($P = 0.080$) and at 4 min of recovery ($P = 0.054$).

Figure 4.8 Blood lactate concentration ($\text{mmol}\cdot\text{l}^{-1}$). Points are means \pm SD; $n = 10$. * Denotes time point in the glycerol trial significantly different from rest values ($P < 0.050$). § Denotes time point in the water trial significantly different from rest values ($P = 0.001$). † Denotes time point in the glycerol trial significantly different from time point in the water trial ($P < 0.050$).



Discussion

The purpose of this study was to examine muscle function during isometric exercise at high intensity following glycerol ingestion, increased extracellular osmolality and altered body water distribution.

Skeletal muscle water accounts for 75% of total intracellular water compartment (Hamilton et al., 1993) and muscle cells behave as a perfect osmometer (Bozler, 1965). Exposure of the muscle cells to hypertonic media has been shown to lead to shrinkage and loss of water (Blinks, 1965) as water moves freely between the intracellular and extracellular space (interstitial space and plasma) and any changes in osmolality will result in a water movement. Hamilton et al. (1993) studied the resultant water shifts in perfused cat muscle following acute increases

in plasma osmolality by 17 to 129 mosmol·kg⁻¹ using various volumes of saline and sucrose. Their results showed that plasma volume rose linearly with increasing osmolality and this was entirely at the expense of intracellular muscle water, as interstitial volume did not change. Based on their findings, they suggested that for the average man, an increase in plasma osmolality by 30 mosmol·kg⁻¹ would decrease skeletal muscle water by 1 – 2 l. Lindinger et al., (2011) showed that even a 5% increase in external osmolality may reduce intact-muscle hydration by a small, but significant ~3%. In the present study, glycerol ingestion raised serum osmolality by 16 mosmol·kg⁻¹ (6%), which led to a 7.8% increase in plasma volume. This was attributed to a net increase of water in the plasma (since red blood cell volume was not different among trials), as red blood cell volume remained unchanged when plasma osmolality increased (Buono & Faucher, 1985; Lindinger et al., 1994). It is possible that the increase in plasma volume was associated with a decrease in muscle cell hydration.

The potential change in muscle water did not have any effect on knee extensor muscle strength, as participants produced similar force in the two main trials. These findings agree with data from earlier studies, which indicated that hypohydration does not affect muscle strength (Bigard et al., 2001; Greenleaf et al., 1967; Greiwe et al., 1998; Montain et al., 1998). In the literature, muscle dehydration and its effects on strength have been studied following total body dehydration, where water is lost from both intracellular and extracellular spaces (Guyton & Hall, 1996) and body mass is reduced. Various means (exposure to sauna, exercise combined with heat stress, fluid and caloric restriction) were used to elicit dehydration and the results on the effects of hypohydration on strength are inconclusive. Heat exposure and exercise as a means of dehydration causes core temperature to rise, which has been shown to limit work capacity and promote fatigue during high intensity cycling (Febbraio et al., 1994), but it may well affect muscle function during strength exercise (Baar, 2006; Hedley et al., 2002). In addition, if a participant is not allowed to fully recover following exercise-induced dehydration, the effects of hypohydration cannot be separated from the

performance reducing effects of muscle fatigue (Judelson et al., 2007b). Fluid and caloric restriction may affect muscular endurance but maximum strength does not seem to be affected (Walberg - Rankin et al., 1996). Overall body hypohydration seems to have a limited role in reducing muscle strength and power (Judelson et al., 2007b). In the present study there was not any reduction in total body water, but an alteration of body water distribution due to increased serum osmolality and a possible decrease in muscle water. Although it was observed that the participants experienced a greater difficulty in completing the exercise protocol during the glycerol trial in the present study (perceived effort was not recorded), any degree of muscle water change in this trial did not affect muscle strength during the 12 high intensity isometric contractions.

The strength results from the present study do not verify the in vitro findings on cell and muscle shrinkage, which demonstrated that reduced muscle water reduced muscle contractility. Gulati and Babu (1982) showed that in intact frog muscle fibres, the force of isometric contraction decreased linearly with increasing extracellular tonicity. These results were later confirmed by the findings of Bressler and Matsuba (1991) who demonstrate that maximum isometric tension of single muscle fibres increased when the fibres were exposed to a hypotonic Ringer solution. This was attributed to a greater force produced per cross - bridge. In vitro, reduced cellular hydration and cell shrinkage is accompanied by a potassium efflux through the opening of potassium channels, and intracellular potassium concentration decreases (Haussinger et al., 2004). Additionally, potassium exits the cell during muscle contractions. Under normal conditions, potassium concentration in the cytosol is greater than in the extracellular fluid. As potassium is a charged solute, any changes in potassium concentration may affect the electrical potential of the cell membrane and its excitability (Cairns & Lindinger, 2008). Sjogaard et al., (1985) suggested that a loss of intracellular potassium and an increase in extra cellular potassium concentration might disturb the membrane electrochemical potential and decrease muscle contractility. In the current study, electrolytes were not measured, but participants were able to produce similar force

in both trials indicating that any changes in potassium concentration in the hyper-osmotic glycerol trial were not enough to alter muscle function. Participants were also able to maintain their strength throughout the exercise session.

During short, high-intensity isometric exercise, energy is derived from the ATP – phosphocreatine system and anaerobic glycolysis, which is accompanied by cellular acidification and lactate production (Ahlborg et al., 1972; Katz et al., 1986; Lindinger et al., 1995). Isometric contraction at ~75% MVC causes phosphocreatine concentration to decline by 25% within 5 s (Hultman & Sjöholm, 1983) and this is marked by an impressive rise in inorganic phosphate (Pi) and creatine (Mougios, 2006). Creatine and Pi are intracellular osmolytes that have been shown to pull water into skeletal muscle during muscle contraction (Raja et al., 2006). Lactate is produced by anaerobic glycolysis and accumulated in the muscle during repetitive exercise at high intensity, which in turn leads to an osmotic gradient that favours movement of water into the intracellular space (Sjøgaard et al., 1985; Raja et al., 2006; Bangsbo et al., 1994). Its accumulation in the muscle increases the number of osmotically active particles, and the intracellular water change is highly correlated with increase of lactate (Fitts, 1994; Lindinger, 2005). In the present study, there was a water shift into the non-vascular space during exercise, which was reflected by a decrease in plasma volume. In the two trials, plasma volume during exercise decreased in a similar fashion, by 7.4% in the water trial and 7.5% in the glycerol trial, which are similar to the 7.3% decrease in plasma volume reported by Robergs et al. (1991) during single leg knee extension resistance exercise at 70% of 1 RM. Raja et al. (2006) showed that during a wrist–flexion exercise, intracellular water increased by 7 – 8% at 90% maximum power output and by 12.6% at 100% maximum power output, and it was reported that the predominant factors causing intracellular water increases during short-term, high-intensity exercise were phosphocreatine hydrolysis, intracellular acidification and increased lactate concentration.

In the present study, blood lactate was measured before, during and after exercise. Participants performed 12 isometric efforts at 90% MVC and despite the high intensity and volume of exercise, there was no high blood lactate accumulation (Glycerol trial $1.44 \pm 0.48 \text{ mmol}\cdot\text{l}^{-1}$; Water trial $1.16 \pm 0.84 \text{ mmol}\cdot\text{l}^{-1}$). One reason for this may be that each isometric contraction was only 6 s long; it is generally accepted that during short contractions (<10 s) the majority of energy derives from the phosphocreatine – ATP system (Hultman & Sjöholm, 1983), although the glycolytic system has been shown to contribute to ATP production even during a single 6-s sprint (Gaitanos et al., 1993). Furthermore, muscle blood flow has been shown to be occluded during isometric contractions in excess of 20% MVC (Edwards et al., 1973; Katz et al., 1986) and lactate is poorly removed from the muscle (Harris et al., 1981), as lactate appearance in the blood relates to its concentration gradient between the cytosol and the blood, and to blood flow in the exercising muscles (Mougios, 2006). Intramuscular lactate concentration has been shown to be twice the concentration of blood lactate (Robergs et al., 1991; Sjøgaard & Saltin, 1982) and can be used as a substrate for muscle glycogen synthesis during the recovery period following exercise (Fournier et al., 2002; Robergs et al., 1991). Another factor for the low blood lactate values obtained during this study is the great distance between the exercising muscles and the blood sampling point on the forearm; lactate is known to quickly diffuse into other tissues (non-exercising muscles, the heart, the liver) (Brooks & Gaesser, 1980) and it is likely that some of the lactate released into the blood from the exercising muscles was absorbed by other tissues before reaching the sampling point.

Statistical analysis of blood lactate did provide some interesting findings on cell metabolism despite the small changes of blood lactate over time. On the water trial, blood lactate increased slightly during exercise and then returned to baseline. On the glycerol trial, blood lactate increased following glycerol ingestion and remained higher than baseline throughout the trial. Furthermore, blood lactate on the glycerol trial was higher compared with lactate on the water trial at 15 and 30 min into the recovery period. These findings indicated altered metabolism during

the hyper-osmotic glycerol trial. Following exposure of cells to a hypertonic environment and the subsequent cell shrinkage, regulatory volume increase (RVI) mechanisms are activated to increase intracellular osmolality, draw water back into the cell and re-establish homeostasis (Lang et al., 1998). RVI is accomplished by ion uptake (Na^+ uptake, which is later exchanged for K^+ via the Na^+ - K^+ -ATP pump) and by cellular generation of organic osmolytes (Strange, 2004), such as lactate. Degradation of glycogen to glucose phosphate increases the number of osmotically active particles and thus increases intracellular osmolality (Lang et al., 1998) and glycogenolysis leads to cellular accumulation of lactate. Hence, lactate production in the cell can occur in an effort to increase the number of intracellular particles and osmolality. Additionally, a greater accumulation of intramuscular lactate would also result in a faster efflux of lactate into the blood due to the greater concentration gradient between the muscle and the blood. The findings of the present study during the glycerol trial are in agreement with in vitro observations that exposure of cells to a hypertonic medium increases the release of lactate (Antolic et al., 2007; Lang et al., 1989), as the acidification accompanying intracellular lactate production acts to preserve cell volume (Usher – Smith et al., 2009) via subsequent activation of Na^+/H^+ exchange, Na^+ uptake (Lang et al., 1989) and cell swelling. It can be speculated that the difference in lactate production between the two trials may be due to a greater intracellular lactate accumulation, and its osmotic effect might have partly contributed to the fact that plasma volume in the glycerol trial did not return to its pre-exercise level following exercise, despite a high serum osmolality.

Blood glucose was also monitored during this study. Whilst blood glucose on the water trial did not change over time, on the glycerol trial it had increased at 4 min after exercise and remained higher than baseline throughout the recovery period. Blood glucose on the glycerol trial was also higher than blood glucose on the water trial at 15 and 30 min of the recovery period. One reason why blood glucose may have increased is that during recovery from strenuous exercise the availability of blood glucose, originating from the liver, facilitates delivery of glucose to the

muscles in order to replenish muscle glycogen. Furthermore, liver cells have been shown in vitro to release glucose (following glycogen degradation) when exposed to a hyper-osmotic environment (Lang et al., 1989). In vivo, Berneis et al., (1999) investigated the effects of hyper-osmolality ($296 \text{ mosmol} \cdot \text{kg}^{-1}$) on whole body glucose kinetics and reported increased plasma glucose concentrations due to an increased hepatic glucose production, which can be compared with the results of the hyper-osmotic conditions of the present study. Clinical evidence in diabetic populations also suggests that dehydration and hyperosmolarity are accompanied by insulin resistance (Haussinger et al., 2004) leading to increased blood glucose concentrations. Overall, the fact that increases in blood glucose was observed only on the hyper-osmotic glycerol trial and in combination with the results on blood lactate, suggests that muscle metabolism was likely altered and that there was possibly an extra physiological strain on the exercising muscles during the hyper-osmotic trial.

Despite different body water distribution between conditions, indicated by the haematological indices measured, there was not any detectable difference in muscle function as participants produced similar forces in both trials. Blood lactate values indicated altered muscle physiology, but this was not enough to affect isometric exercise performance. Judelson et al. (2007b) also failed to find any acute measurable changes in isometric back squats after 2.5% and 5.0% body mass hypohydration, but further research showed that chronic hypohydration could have a different effect as it produces a less beneficial postexercise milieu, increasing catabolism and potentially decreasing anabolism (Judelson et al., 2008). In general, moderate to high intensity resistance exercise acutely increases protein synthesis (Baar, 2006; Tipton & Wolfe, 2001). In the process, amino acid (Tipton & Wolfe, 2001) availability provides a more favourable environment for protein synthesis. Muscle increases are mediated by action of anabolic hormones such as testosterone and insulin (Greig et al, 2006). Testosterone levels increase in the recovery period after a strength session (Kraemer et al., 1998), but exercise in a hypohydrated state seemed to blunt the

testosterone response (Judelson et al., 2008). Insulin not only controls blood glucose, but it also facilitates amino acid entry into the cell, which may promote protein synthesis (Mougios, 2006), and insulin action leads to cell swelling which also can stimulate protein synthesis (Haussinger et al., 2004). A decrease in cell volume caused by dehydration due to exposure to a hyper-osmotic environment promotes insulin resistance (Schliess & Haussinger, 2003; Schliess et al., 2001), negatively affecting protein synthesis during the recovery period. In addition, performing strength exercise in a hypohydrated state can increase circulating cortisol levels which will promote catabolic effects, suggesting that hypohydration significantly enhances the stress of resistance exercise (Judelson et al., 2008) and this additional stress might hinder the body's ability to recover from exercise.

Having failed to find any acute effects of glycerol ingestion and altered body water distribution on high-intensity strength exercise, future research would be directed into examining the potential effects of long-term exercise training and recovery under high extracellular osmolality and altered body water distribution.

Chapter 5

Effect of glycerol ingestion during an eight-week strength training period

Introduction

In previous work reported in the two preceding chapters, acute changes in body water distribution, as expressed by an increase in plasma volume and potentially a decrease in intracellular water, did not appear to have an effect on muscular function and force production during high-intensity isometric exercise. Although there are no studies examining long-term effects of muscular hypohydration on muscle adaptations to strength training, evidence suggests that hypohydration may attenuate some benefits of a resistance exercise programme (Judelson et al., 2007b). Therefore, the next area to be investigated was whether there are any long-term effects of exercise training following glycerol ingestion and presumed altered body water distribution.

Resistance training improves strength and causes adaptations in the skeletal muscle, such as an increase in cross-sectional area (Maughan et al., 1984a). Gains in muscle size and strength are elicited by training programmes of high loading at intensities greater than 75% of 1RM (Chestnut & Docherty, 1999). Training at intensities lower than 50% of 1RM does not seem to induce any hypertrophy (Moss et al., 1997). Induced skeletal muscle hypertrophy produced by strength exercise of short duration and high intensity (Booth & Thomason, 1991) has been linked to acute changes in the cellular processes involved in protein turnover and muscle growth (Tipton & Wolfe, 2001) following an exercise session; increased serum concentration of testosterone, growth hormone and IGF-1 (all of which have an anabolic effect on protein synthesis) (Crewther et al., 2006) and increased protein synthesis, which exceeds protein breakdown (Mougios, 2006), all interact to regulate muscle protein remodelling following heavy resistance exercise (Kraemer et al., 1998).

Muscle metabolism and protein synthesis can be affected by cellular hydration (Haussinger et al., 1994). In vitro, it has been shown that exposure of cells to a hyper-osmotic environment leads to cellular hypohydration and this promotes

catabolism (Lang et al., 1998). Body hypohydration has also been shown to modify the metabolic response to resistance exercise (Judelson et al., 2008) by decreasing testosterone and insulin secretion and increasing circulation cortisol concentrations. This potentially alters the anabolic processes taking place in the post-exercise recovery period, and may have an impact on the expected training outcome if an athlete exercises under a hypohydrated state for an extended period of time.

For the purpose of this study, extracellular osmolality was altered by the ingestion of a hyper-osmotic glycerol solution. Since the cells function as an osmometer (Haussinger et al., 1994; Strange, 2004) the increased extracellular osmolality may result in water efflux from the intracellular space, as skeletal muscle fibres have been shown to adjust their volume in response to alterations in the osmolality of the extracellular environment (Blinks, 1965).

In the present study, a training protocol typically used to produce hypertrophy (McCaulley et al., 2009; Smith & Rutherford, 1995; Schott et al., 1995; Yamamoto et al., 2008) was employed to exercise the forearm muscles. One more issue was the choice of type of exercise. The use of multi-joint exercises using big muscle groups would make the interpretation of results difficult with regard to the exact loading of the muscle and gains in size and strength of each muscle. Measuring acute and chronic volume changes of big muscle groups would also pose a greater challenge than measuring a smaller area. Therefore, handgrip exercise was chosen as a more practical way for examining acute and long term changes in muscle volume and strength. Additionally, each exercise effort would be easily monitored and, with appropriate feedback, the trainees would be more able to maintain the prescribed exercise intensity.

The aim of this study was to investigate whether exercising and initiating recovery under an altered body water distribution would have an effect on forearm muscle volume and strength after 8 weeks of handgrip training at high intensity.

Materials and Methods

Following ethical approval by the Loughborough University Ethical Advisory Committee, fourteen healthy male volunteers participated in this study. After a written and oral explanation of all procedures, and their right to withdraw at any point, each participant signed a written consent form. All participants ($n = 14$) were physically active and accustomed to resistance exercise. Their age, height and body mass were, respectively, (mean \pm SD) 27 ± 4 y, 174 ± 6 cm, and 73.6 ± 5.1 kg.

Experimental procedure

Participants were randomly assigned to one of two training groups. The first group ingested lemon-flavoured water ($6 \text{ ml} \cdot \text{kg}^{-1}$ body mass) during the training sessions and the second group ingested a lemon-flavoured glycerol solution (total volume $6 \text{ ml} \cdot \text{kg}^{-1}$ body mass, $1 \text{ g} \cdot \text{kg}^{-1}$ body mass glycerol). Half the drink volume was ingested 30 min before exercise and the remaining half was ingested immediately after exercise.

Training

Following familiarisation with the protocol, participants trained their stronger arm three times a week (on a Monday, Wednesday and Friday) for eight weeks by performing four sets of 10 repetitions (sustained for 1 s) at 80% of their maximum handgrip strength using a Jamar® Hydraulic Hand Dynamometer, with 2 minutes rest between sets. The other arm acted as a control. Participants trained on alternate days, at the same time of day. In order to maintain the appropriate intensity, during training sessions, participants were given oral feedback regarding the force they were generating with each repetition. During all training sessions and maximum handgrip tests, participants sat in an adjustable, straight-backed chair. The exercising arm was placed by the side of the body, with the elbow extended, and the forearm and wrist held in a neutral position.

Maximum handgrip strength test and forearm volume

Three days before the start of the training period, participants visited the laboratory for an initial assessment of their maximum handgrip strength of both arms, using the same apparatus that would be used during the training sessions. The best of three efforts was taken as their maximum. There was one minute of rest between efforts. When evident that a participant was improving with every repetition, he proceeded to perform more efforts, until a true maximal value was obtained. Maximum handgrip strength for both arms was assessed every two weeks, and absolute training intensity was adjusted so it always corresponded to 80% maximum handgrip strength. Following completion of the 8-week training period, participants visited the laboratory ~72 hours after the last training session for the last maximum handgrip strength measurement. A 72-h period was given to allow full recovery from the last training session.

Forearm volume was measured in duplicate by the water displacement method (CV 1.4%) as described by Maughan et al. (1984b), in order to measure any changes in forearm muscle volume during the eight-week training period. Forearm volume of both arms was measured before the training period, every two weeks during the training period, and at the end of the training period. Acute changes in forearm volume of the exercising arm were measured at the first and last training sessions, before and immediately after the four sets of handgrip exercise.

All training sessions lasted 45 min, apart from the first and last sessions which involved blood sampling, and therefore lasted three hours. For the long sessions that included blood sampling, participants came to the laboratory after an overnight fast. After voiding, body mass was measured and participants were seated comfortably in the laboratory in the same upright chair that was used in all of the training sessions. The non-exercising arm was immersed in water (42°C) for 10 min. Then, a small canula (butterfly, size 21g) was inserted into a superficial vein. The canula stayed in place throughout the session to allow multiple 3 ml blood samples to be drawn. Two resting blood samples were obtained 10 min apart. Immediately after the second sample, participants ingested

half the volume of the test drink. Another blood sample was drawn 30 min after ingestion and exercise started. Blood samples were also drawn at the end of each set and then at 15, 30, 45, 60 and 90 min, post-exercise.

Forearm volume of the exercising arm was measured during rest before exercise and immediately after exercise.

Sample analysis

Blood samples were analysed for haemoglobin concentration, haematocrit, blood glucose and blood lactate. Haemoglobin concentrations and haematocrit were used to estimate changes in blood, red cell and plasma volumes as described by Dill and Costill (1974).

Serum samples were analysed for osmolality and glycerol.

All analyses were performed as described in the general methods section of this thesis.

Statistical Analysis

Changes in strength, forearm volume, blood glucose and lactate, serum osmolality and glycerol were analysed using repeated measures ANOVA for group, time and “group x time” interaction. Changes over time were analysed using one-way ANOVA. Where appropriate, significant differences were detected using a Tukey post-hoc test.

Data not normally distributed were analyzed with non-parametric statistical tests.

Results

Independent t-test comparing the values obtained prior to the training period showed that there was no difference in handgrip strength ($P = 1.000$) and forearm volume ($P = 0.535$) of the exercising arm between the two training groups. Therefore, the two groups were equally matched in handgrip strength and forearm volume (Table 5.1).

Table 5.1 Participant characteristics (means \pm SD) at the beginning of the training period (Water group $n = 7$, Glycerol group $n = 7$). P denotes comparison between groups.

	Water Group	Glycerol Group	P value
Age (y)	27 ± 3	27 ± 5	0.901
Height (cm)	173 ± 6	176 ± 5	0.379
Body mass (kg)	72.2 ± 5.2	75.0 ± 4.9	0.316
Dominant arm handgrip strength (kg)	51 ± 4	51 ± 8	0.933
Non dominant arm handgrip strength (kg)	48 ± 5	48 ± 6	0.963
Dominant arm forearm muscle volume (ml)	781 ± 94	812 ± 68	0.494
Non dominant arm forearm muscle volume (ml)	767 ± 79	803 ± 72	0.388

Blood analysis

The analyses described in this section are from the blood samples taken during the first training session. Analysis of blood samples over time describes changes within a single training session.

Serum glycerol concentration (Table 5.2)

Two-way repeated measures ANOVA showed a group effect ($P = 0.000$), time effect ($P = 0.000$) and an interaction ($P = 0.000$). Post-hoc analysis showed no difference in serum glycerol between the two groups at the rest point prior to glycerol or water ingestion ($P = 0.515$). In the glycerol group, following glycerol ingestion, serum glycerol increased ($P = 0.000$) to $7.41 \pm 2.34 \text{ mmol}\cdot\text{l}^{-1}$ and remained higher than the resting values ($P < 0.001$) and higher than all the points of the water group throughout the experimental trial ($P = 0.000$). One-way ANOVA analysis over time for the water group showed no change in serum glycerol ($P = 0.500$).

Table 5.2 Serum glycerol concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD (Water group $n = 7$, Glycerol group $n = 7$). P denotes difference between groups.

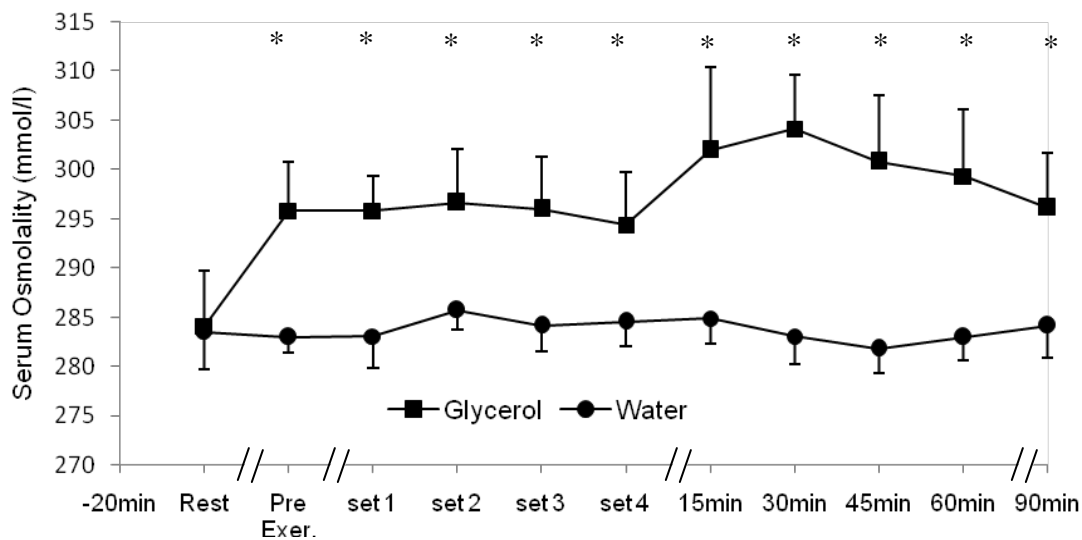
	Rest	Pre Exercise	Set 4 End Exercise	15 min	30 min	45 min	60 min	90 min
Water group	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
Glycerol group	0.03 ± 0.01	7.41 ± 2.34	6.45 ± 2.03	11.09 ± 4.74	12.62 ± 2.83	10.21 ± 1.95	8.88 ± 1.33	7.26 ± 1.46
P	0.515	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Serum Osmolality (Figure 5.1)

Two-way repeated measures ANOVA showed a group effect ($P = 0.003$), time effect ($P = 0.000$) and an interaction ($P = 0.000$). At rest, there was no difference in serum osmolality between the two groups ($P = 0.859$). One-way ANOVA over time showed that following the initial glycerol ingestion, serum osmolality increased ($P = 0.040$) to $296 \pm 5 \text{ mosmol}\cdot\text{kg}^{-1}$ in the glycerol group. After exercise,

following ingestion of the second portion of the glycerol solution, osmolality increased further ($P = 0.000$) to 304 ± 5 mosmol \cdot kg $^{-1}$ and remained elevated compared with the resting value ($P < 0.030$). Post-hoc analysis showed that following glycerol ingestion serum osmolality in the glycerol group was higher than osmolality in the water group throughout the session ($P < 0.002$).

Figure 5.1 Serum osmolality (mosmol \cdot kg $^{-1}$). Points are means \pm SD; Water group $n = 7$, Glycerol group $n = 7$. * denotes glycerol time point significantly different ($P < 0.050$) from water point and glycerol resting point.



Plasma volume changes (Figure 5.2)

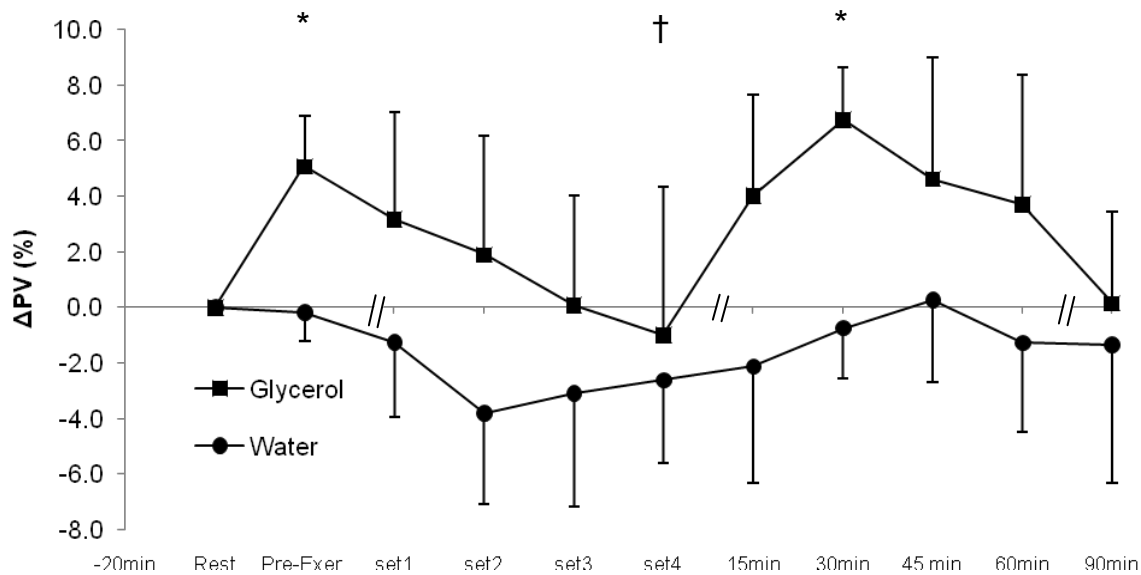
Two-way repeated measures ANOVA showed a group effect ($P = 0.021$). Time tended to have an effect on plasma volume ($P = 0.055$), and there was no interaction ($P = 0.220$). Post-hoc analysis showed that during the initial resting period following glycerol ingestion plasma volume increased by $5.2 \pm 3.3\%$ ($P = 0.009$) in the glycerol group.

During exercise, plasma volume in the glycerol group decreased compared with the pre-exercise point by $5.7 \pm 5.0\%$ ($P = 0.023$). In the water group, plasma

volume tended to decrease ($2.8 \pm 2.8\%$) during exercise, but it did not reach statistical significance ($P = 0.079$).

Following the second glycerol ingestion after exercise, plasma volume increased above resting values reaching a maximum increase of $6.1\% \pm 1.1$ at 30 min ($P = 0.042$) (Figure 5.2).

Figure 5.2 Estimated percentage plasma volume changes. Points are means \pm SD; Water group $n = 7$, Glycerol group $n = 7$. † denotes glycerol group exercise point significantly different from pre-exercise point ($P < 0.050$). * Denotes glycerol point higher than rest point ($P < 0.050$)



Blood glucose concentration (Table 5.3)

Two-way repeated measures ANOVA showed no group effect ($P = 0.722$), a time effect ($P = 0.000$) and no interaction ($P = 0.080$). Post-hoc analysis showed that there was no difference in blood glucose between the two groups at rest ($P = 0.861$). Time pair wise comparisons showed that there was a difference in glucose between the 90 min point and set 1 ($P = 0.038$), 90 min point and set 4 (end of exercise) ($P = 0.005$).

One-way ANOVA analysis over time within groups showed that compared with the resting values, blood glucose did not change during the experimental trials ($P > 0.05$) in either of the groups. Post-hoc analysis showed that blood glucose was not different between the two groups ($P > 0.050$) at any point.

Table 5.3 Blood glucose concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; Water group $n = 7$, Glycerol group $n = 7$. P denotes difference between the two groups.

	Rest	Pre Exercise	Set 1	Set 2	Set 3	Set 4	15 min	30 min	45 min	60 min	90 min
Water group	5.06 ± 0.41	5.10 ± 0.62	4.92 ± 0.52	4.94 ± 0.44	5.02 ± 0.53	4.93 ± 0.47	4.98 ± 0.57	5.14 ± 0.51	5.27 ± 0.48	5.39 ± 0.42	5.65 ± 0.87
Glycerol group	5.03 ± 0.68	5.34 ± 0.87	5.09 ± 1.08	5.19 ± 1.02	5.06 ± 1.05	4.96 ± 1.06	5.13 ± 0.85	5.10 ± 0.93	5.14 ± 0.94	5.28 ± 0.92	5.37 ± 1.11
P	0.921	0.590	0.746	0.598	0.949	0.950	0.725	0.939	0.766	0.785	0.629

Blood lactate concentration (Fig. 5.3)

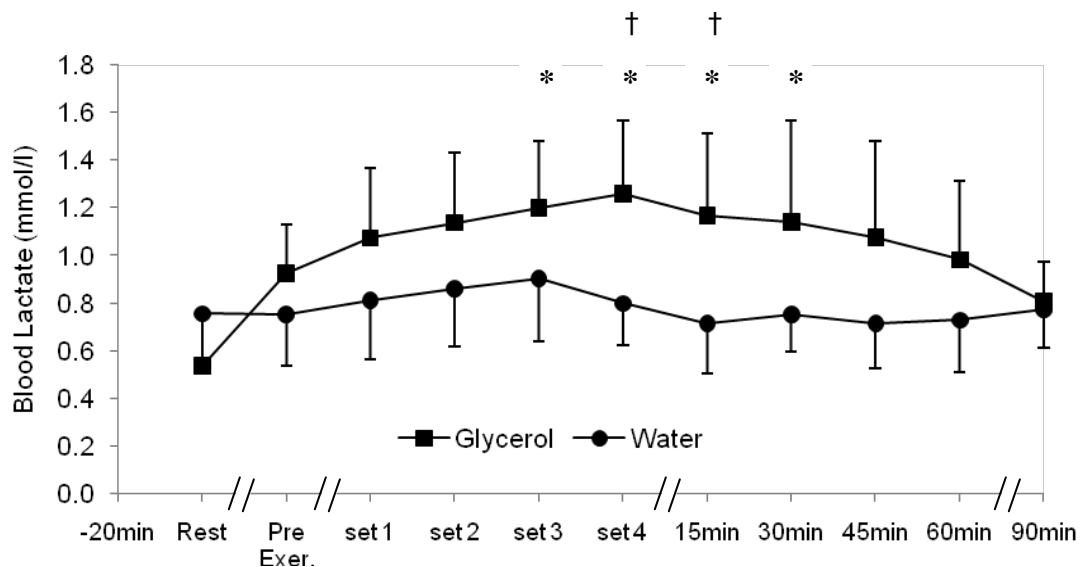
Two-way repeated measures ANOVA showed no group effect ($P = 0.173$), a time effect ($P = 0.004$) and an interaction ($P = 0.045$). Post-hoc analysis showed that there was no difference in blood lactate between the two groups at rest before glycerol ingestion ($P = 0.253$) or after ($P = 0.728$).

One-way ANOVA analysis over time within groups showed no change in blood lactate in the water group ($P = 0.900$). For the glycerol group, one-way ANOVA analysis over time showed that compared with the initial rest point, blood lactate was higher ($P < 0.05$) at set 3, set 4, 15 min and 30 min into recovery.

Between groups, comparisons during exercise showed that blood lactate was higher in the glycerol group after set 4 (end of exercise) ($P = 0.012$). Following the

second ingestion of glycerol, blood lactate was higher in the glycerol group at 15 min ($P = 0.022$). At 30 min, the difference between groups was $P = 0.065$, and at 45 min, $P = 0.076$.

Figure 5.3 Blood lactate concentration ($\text{mmol}\cdot\text{l}^{-1}$). Points are means \pm SD; Water group $n = 7$, Glycerol group $n = 7$. * Denotes difference compared with rest point in glycerol group ($P < 0.05$). † denotes glycerol time point significantly different ($P < 0.050$) from water point.

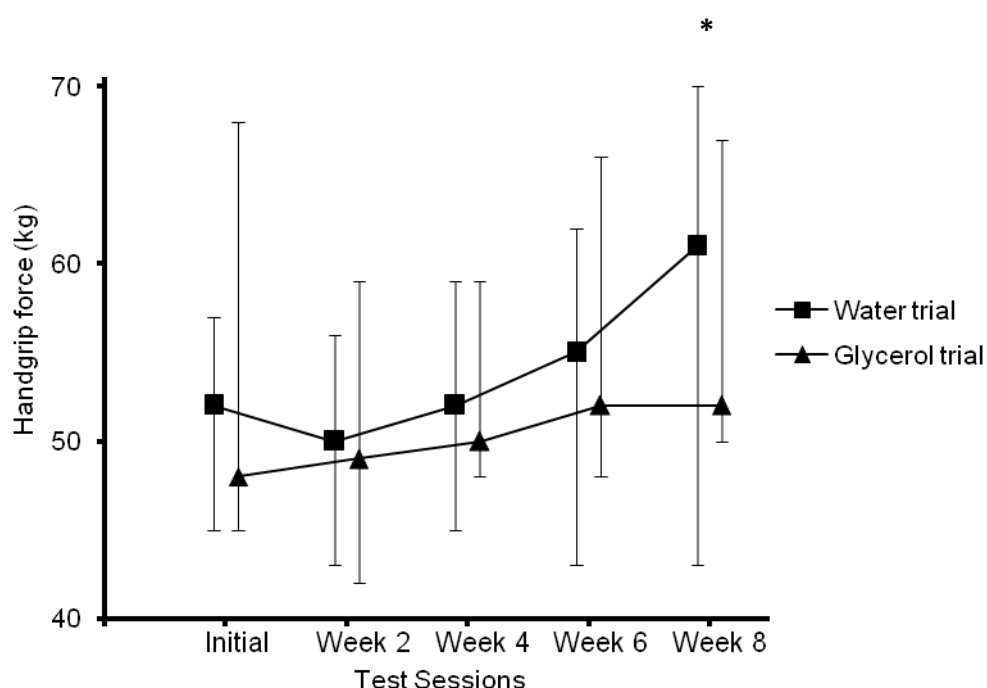


Handgrip strength (Fig. 5.4)

As shown in table 5.1, there was no difference between groups in their maximum handgrip strength prior to the training period. A Shapiro-Wilks test showed that data on handgrip strength were not normally distributed ($P < 0.01$) and handgrip data were analyzed with non-parametric statistical tests. A Friedman test showed that there was a time effect ($P = 0.026$), but no trial effect ($P = 0.340$) and no interaction ($P = 0.800$). Overall, handgrip strength increased over the 8 weeks of training ($P = 0.030$; Kruskal-Wallis test) but there was no difference between groups.

In the water group, handgrip strength increased by 8 ± 6 kg (16%) to 59 ± 8 kg (median 61 kg, range 48 – 70 kg), and in the glycerol group, by 4 ± 4 kg (6%) to 54 ± 6 kg (median 52 kg, range 50 – 67 kg). The large variation in strength improvements between participants and the small number of participants might have prevented the values in handgrip strength from reaching a statistically significant difference between groups.

Figure 5.4 Handgrip strength (kg) for the exercising arm before and after training period. Values are median (range); Water group $n = 7$, Glycerol group $n = 7$. * denotes handgrip value (for both groups combined) after the training period higher than initial pre-training assessment ($P < 0.050$).



Forearm volume – Exercising arm (Fig. 5.5)

Forearm volume prior to the training period was not different between groups, as shown in table 5.1. Forearm volume did not measurably change over the 8-week training period (Table 5.4).

Two-way repeated measures ANOVA over the 8-week training period showed that there was no group effect ($P = 0.552$), no time effect ($P = 0.520$) and no interaction ($P = 0.947$).

Results within group paired t – tests during a single training session showed that compared with forearm volume at the resting period, at the end of exercise (set 4), forearm volume increased by 20 ± 7 ml (2.8%) in the water group ($P = 0.001$) and by 17 ± 11 ml (2.1%) in the glycerol group ($P = 0.006$).

Figure 5.5 Acute forearm muscle volume (ml) before and immediately after 4 sets of handgrip exercise. Values are means \pm SD; Water group $n = 7$, Glycerol group $n = 7$. * denotes difference from pre-exercise point ($P < 0.010$).

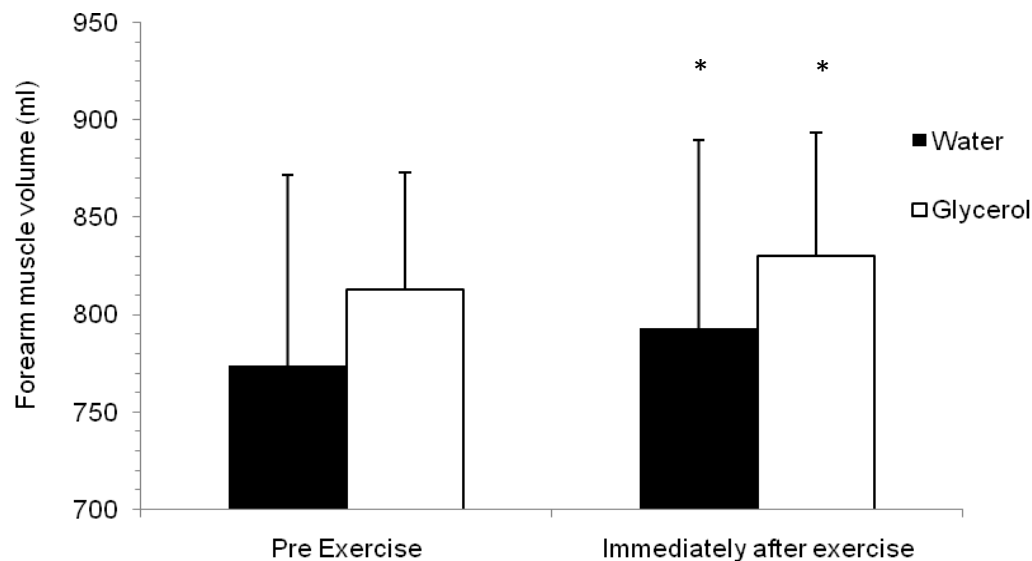


Table 5.4 Forearm muscle volume (ml) before and after 8 weeks of handgrip training. Values are means \pm SD; Water group $n = 7$, Glycerol group $n = 7$. P denotes difference over time within group.

	Before Training	After 8 weeks of training	P
Water group	781 \pm 94	783 \pm 99	0.875
Glycerol group	812 \pm 68	817 \pm 72	0.661

Non-exercising control arm data

Handgrip strength

Two-way repeated measures ANOVA on handgrip strength for the control arm showed that there was no group effect ($P = 0.684$), no time effect ($P = 0.163$) and no interaction ($P = 0.460$), as handgrip strength for the control group did not change over time in either of the two groups. For the water group, handgrip strength was 48 ± 5 kg at the beginning of the study and 50 ± 5 kg at the end of the training period. For the glycerol group, handgrip strength at the beginning of the study was 48 ± 6 kg and at the end of the study period, handgrip strength was 50 ± 5 kg.

Forearm muscle volume

Two-way repeated measures ANOVA on forearm muscle volume for the control arm showed that there was no group effect ($P = 0.552$), no time effect ($P = 0.610$) and no interaction ($P = 0.664$), as forearm volume did not change over time in either of the two groups. For the water group, forearm muscle volume was 767 ± 79 ml at the beginning of the study and 752 ± 81 ml at the end. For the glycerol group, forearm muscle volume was 803 ± 72 ml at the beginning of the study and 798 ± 82 ml at the end of the study.

Discussion

Resistance training is widely accepted as an important tool to increase muscle strength and muscle volume (i.e., hypertrophy). Type of training (Crewther et al., 2006; Folland & Williams, 2007), volume and intensity of exercise (Kanehisa et al., 2002; McCaulley et al., 2009) and training status (Maughan et al., 1984a; Crewther et al., 2006) can all influence the outcome of resistance training programmes. Body hydration status has also been shown to alter the hormonal response to resistance exercise, namely decreasing the anabolic hormone testosterone and increasing the catabolic stress hormone cortisol, potentially altering the training effect and adaptation of exercising muscles (Judelson et al., 2007b). The aim of this study was to investigate potential differences in strength and muscle volume following eight weeks of strength training at high intensity, under potentially different body water distribution status. The participants in this study were recreationally active individuals but no one engaged in regular weight training. Therefore, they were considered untrained and suitable for this study, as changes in untrained individuals are greater than trained participants, and easier to monitor (Crewther et al., 2006).

The glycerol group ingested the first bolus of the high concentration glycerol solution prior to exercise. Following the initial glycerol ingestion, serum glycerol increased; this was accompanied by an increase in osmolality of more than 10 mosmol·kg⁻¹, and an increase of plasma volume by 5.2% prior to exercise. As already discussed in the previous chapters of this thesis, the changes in extracellular osmolality induced presumably a modest state of cell shrinking (Keller et al., 2003), since in vitro data suggest that increases in plasma volume induced by hypertonic fluids may come entirely at the expense of cell volume (Hamilton et al., 1993). The presumed reduced muscular hydration did not affect exercise, as participants in the glycerol group were able to exercise at the required intensity of 80% MVC, as mild to moderate levels of hypohydration does not seem to affect

strength acutely (Bigard et al., 2001; Greenleaf et al., 1967; Greiwe et al., 1998; Montain et al., 1998).

During exercise, plasma volume decreased on the glycerol group and tended to decrease on the water group, whilst forearm muscle volume increased in both groups by the end of exercise. These changes demonstrate the water shift and increase in muscle fluid, which occurs during muscle contractions (Nygren et al., 2000; Sjogaard & Saltin, 1982). The significant finding of the present study is that the decrease in plasma volume on the glycerol group was greater than on the water group and it was accompanied by an increase in blood lactate, which was not observed on the water group. The increase in muscle water during contraction is attributed to osmotic pressure changes due to the accumulation of osmolytes such as ADP, inorganic phosphate, creatine and lactate (Rapp et al., 1998). In the type of exercise used in the present study, energy derives from the ATP – Phosphocreatine system (Katz et al., 1986; Lindinger et al., 1995) and a 10 s forearm isometric contraction at 70% MVC has been shown to decrease phosphocreatine by 30% (Kimura et al., 2006), which results in the increase of free inorganic phosphate and creatine. The decrease in plasma volume observed in the present study during exercise on both trials can therefore be attributed to the potential accumulation of metabolic, osmotically active molecules within the contracting muscles, which swells, as solute-free water from the vascular space enters the exercising muscles (Lindinger et al., 2011; Lindinger et al., 1994). The difference between the two experimental groups is that blood lactate increased during exercise only in the glycerol group, suggesting altered muscle metabolism in the glycerol group. Lactate is formed as a product of anaerobic glycolysis derived from either blood glucose or muscle glycogen. In the present study, blood glucose during exercise was neither different from resting values nor different between groups. It may be that the increased lactate observed in the glycerol group derived from increased glycogenolysis. It is possible that the hyper-osmotic stress in the glycerol group led to higher rates of ATP turnover as observed by Cermak et al. (2009) during isometric contraction of muscles exposed

to a 400 mosmol·kg⁻¹ environment, and this led to a shift in substrate utilization favouring a greater glycolytic flux (Hultman, 1995).

Blood lactate in the glycerol group was also higher compared with the water group for the first 30 min of the recovery period. As participants in the glycerol group ingested the second bolus of the glycerol solution immediately after exercise, serum osmolality increased to 304 mosmol·kg⁻¹ and plasma volume increased by 6.1% compared with the resting value. Plasma volume was higher than baseline for a period of 30 min after exercise, possibly demonstrating that participants recovered from exercise in a state of altered body water distribution. Increased lactate production in resting conditions has also been reported by Antolic et al. (2007); following exposure of whole muscles to a hypertonic bath (400 mosmol·kg⁻¹) and the subsequent cell shrinkage, tissue lactate increased four-fold. The authors suggested that this was due to an effort of the muscle cells to regulate and re-establish cell volume via an increase in intracellular osmolality and an attraction of water back into the cell. Similarly, Lang et al. (1989) showed in vitro that exposure of liver cells to a hypertonic environment resulting in cell shrinkage is accompanied by an increase in lactate release from the muscle. This is in agreement with Usher-Smith et al. (2009) who, in a review of literature, concluded that intracellular lactate and the accompanying acidification acts to regulate and preserve muscle cell volume. In the present study, the increased lactate efflux observed in the glycerol group is an indication that skeletal muscle water was likely reduced during the first 30 min of recovery, which does not favour anabolic processes in the cell (Haussinger, 1996; Haussinger et al., 1994).

As muscle hypertrophy is the result of remodelling of the muscle and the increase in muscle protein synthesis that take place following strength exercise sessions (Crewther et al., 2006; Jones & Rutherford, 1987; Folland & Williams 2007; Tipton & Wolfe, 2001), a potential reduction in cell hydration in the recovery period could lead to altered training effects. In vitro, cell shrinkage has been shown to stimulate protein breakdown (Hallbrucker et al., 1991a; Haussinger et al., 1990; Haussinger

et al., 1991; vom Dahl et al., 1991) and inhibit amino acid uptake (Haussinger, 1996; Haussinger et al., 1994) both of which would decrease protein synthesis and muscle growth (Biolo et al., 1997; Tipton & Wolfe, 2001). In vivo, Berneis et al. (1999) failed to find any changes in whole body protein synthesis or protein breakdown during hyperosmolal conditions ($296 \text{ mosmol} \cdot \text{kg}^{-1}$). Keller et al. (2003) also failed to find any effects of hyperosmolality (induced by intravenous infusion of hypertonic NaCl and restriction of water intake) on protein breakdown. In the present study, the results showed that compared with baseline measurements, there was no change in muscle volume in either of the two groups over the eight weeks of handgrip training.

The type of training in the present study was similar to a widely used mode and volume of training that is expected to induce hypertrophy; 4 sets of 10 repetitions at high intensity ($>75 - 80\% \text{ max}$) (Kraemer et al., 1990; McCaulley et al., 2009; Yamamoto et al., 2008). In the present study, forearm muscle volume was not measured directly, but was estimated using the water displacement method (Maughan et al., 1984b), where the forearm is submerged in a volumetric canister and the displaced water gives the forearm volume. The water displacement method is valid and is also used in the medical/physiotherapy field (Karges et al., 2003). In the present study, it was also sensitive enough to detect acute changes in muscle volume during training sessions. It is important to note that acute increases in forearm volume immediately after a set of muscle contractions may partially be the result of the increased muscle blood flow that has been shown to increase between contractions during a train of contractions (Gonzales et al., 2007), which minimizes the effect of the mechanical compression placed on the muscle vasculature. This is in addition to the water that enters the contracting muscles due to the osmotic gradients set up by the increase in cytosol metabolites generated by the exercise. The absence of muscle hypertrophy at the end of the training period in the present study is puzzling. From the study data it cannot be specified whether the training load used was an insufficient stimulus to induce hypertrophy or whether the training period was too short for this purpose. The

training volume and intensity were similar to that of Cureton et al. (1988) and O'Hagan et al. (1995) who reported muscle hypertrophy after 12 and 20 weeks of training, respectively. Therefore it is likely that had the present study training continued for more than 12 weeks, measurable increases in arm muscle mass would have been evident in at least one of the training groups. (Folland & Williams, 2007; Kanehisa et al., 2002).

Strength increased similarly in both groups in the present study with the water group's maximum handgrip strength improving by ~16% and in the glycerol group by ~ 8%. The small number of participants in each group and the large standard deviation in strength measurements may have prevented the results between groups from reaching statistical significance. A post-hoc power test based on the average strength scores obtained from the present study suggested that with the large standard deviation observed at the end of the training period (± 8 kg the water group; ± 6 kg glycerol group) 25 participants per group would be required in order to have an 80% chance of obtaining measurable differences between groups, whereas a much smaller standard deviation of ± 4 kg would require only 8 subjects per group to yield a statistically significant difference between groups.

It is possible for strength to increase without increases in the cross-sectional area (Jones, 1992). Young et al. (1983) found an increase in muscle strength, which was greater than could be accounted for by a change in the cross-sectional area of the muscle group. In that study, participants exercised the quadriceps muscles three times per week for five weeks. Their maximum isometric strength increased by 15%, while the muscle cross-sectional area increased by only 6%. Jones and Rutherford (1987) examined the effects of isometric quadriceps training at 80% MVC, three times per week, for 12 weeks. Their results showed that isometric force increased by 34%, whilst hypertrophy increased by only 5%, and they reported no correlation between changes in force and the cross-sectional area. The authors concluded that as a result of strength training, the main change in the first 12 weeks is an increase in the force generated per unit cross-sectional area of

muscle, possibly due to increased activation of the muscle as a result of changes in motor unit firing patterns. Indeed, changes in the neuromuscular system may account for most of the increases in force generation in the early stages of training (Hakkinen et al., 1985). Following resistance training, muscle force can be augmented as a result of better synchronization of motor unit contractions (Duchateau & Hainaut, 1984), an increase in the motor units firing frequencies rate (Van Cutsem et al., 1998), an increase in central descending (efferent) motor drive, an increase in motor unit excitability and a decrease in neural inhibition (Aagaard, 2003). During the present study, no data were collected regarding potential neuromuscular changes, and no conclusions can be made regarding this issue.

Overall, there were no significant differences in the training effect between the two training groups in the present study, neither were there any differences for the control arm. The hypertonicity and the presumed altered cell hydration on the glycerol trial did not affect forearm muscle volume. As cell shrinkage results in increased protein breakdown (Haussinger et al., 1994; Lang et al., 1998), it was hypothesized that the training effect could be altered in the glycerol group. Muscle hypertrophy comes as a result of positive net balance; protein synthesis must exceed muscle protein degradation. Resistance exercise also seems to increase muscle protein breakdown, but to a lesser degree than protein synthesis (Tipton & Wolfe, 2001). If muscle degradation were to be increased due to cell hypohydration, muscular development in the glycerol group would be less than in the water group. Muscle protein metabolism was not measured in the present study, but in vivo Yamamoto et al. (2008) did not detect a greater magnitude of muscle damage consequent to resistance exercise in hypohydrated (5% body mass) trained weight lifters, compared with exercise in the euhydrated state. Still, the authors suggested that athletes should exercise in a euhydrated state to maximise the endogenous hormonal and metabolic benefits. Hypertrophy results from exercise of a short duration and high intensity (Booth & Thomason, 1991) as this increases muscle protein synthesis (Tipton and Wolfe,

2001) resulting from a change in the metabolic state of the muscle and the action of various hormones (testosterone, growth hormone, insulin, IGF-1) and growth factors (Baar, 2006). Testosterone and growth hormone increase acutely following resistance exercise (Kraemer et al., 1998) and they contribute to muscle growth by increasing protein synthesis and decreasing protein degradation (Crewther et al., 2006). Resistance exercise also increases secretion and action of insulin-like growth factor IGF-1 (Widerman et al., 2002), which also promotes protein synthesis and decreases protein degradation. Insulin is another hormone related to exercise and muscle metabolism. Insulin's primary role is to regulate blood glucose, but it also has a strong anabolic effect, as it increases the uptake of carbohydrates and amino acids into the muscle (Crewther et al., 2006). Furthermore, insulin induces cell swelling (Vom Dahl et al., 1991) and this increase in intracellular water space is a crucial event in mediating the antiproteolytic effect of insulin (Haussinger et al., 1991). All of the above-mentioned hormones are related to cell swelling (Lang et al., 1998) and their action is inhibited by cell shrinkage. Hypohydration and consequent cell shrinkage may indeed alter the hormonal response to exercise. Judelson et al. (2008) showed that exercising while hypohydrated (decreased total body water) decreased circulating testosterone and raised insulin resistance, in agreement with previous in vitro findings (Schliess & Haussinger, 2000). Furthermore, Judelson et al. (2008) reported that hypohydration increased circulating cortisol; strengthening the hypothesis that hypohydration may have a catabolic effect in vivo. Since hormones were not measured in this study, it cannot be determined whether the training protocol and the difference in body water distribution had any effect on the secretion and action of hormones which affect protein synthesis.

In conclusion, glycerol ingestion and the resultant movement of water into the vascular space and potential mild muscle hypohydration did not have a significant acute or chronic effect on muscle function during an 8-week handgrip strength training period. There were few participants in each group and this might have prevented differences between groups in regards to increases in strength from

reaching statistical significance. However, in general, hypohydration does not seem to affect strength and power (Maughan, 2003).

On the other hand, glycerol ingestion resulted in a significant increase in plasma and blood volume, which can have an effect on aerobic exercise. Increases in blood and plasma volume have been shown to elicit improvements in aerobic performance and increase maximum oxygen uptake ($\text{VO}_{2\text{max}}$) (Warburton et al., 1999; Coyle et al., 1986; Coyle et al., 1990; Hopper et al., 1988). Acute plasma volume and blood volume expansion represents a potential ergogenic aid (Krip et al., 1997). Bearing in mind that the shift of water into the vascular space following glycerol ingestion may possibly compromise the intracellular water volume, a new question arises: Would glycerol ingestion and the subsequent blood volume increase bring any improvements in $\text{VO}_{2\text{max}}$ and aerobic performance, or would a potential decrease in intracellular water inhibit performance?

Chapter 6

Effects of glycerol ingestion and subsequent plasma and blood volume expansion on VO_2max

Introduction

Maximum oxygen uptake (VO_2max) is defined as the highest rate at which oxygen can be taken up by the body and utilised during severe exercise. The current belief is that maximum cardiac output is the principal limiting factor for VO_2max during cycling tests, as the observed normal range of VO_2max in trained men and women of similar age is due principally to variation in maximal stroke volume (Ekblom, 2000; Basset et al., 2000). Stroke volume is affected by blood volume (Hopper et al., 1988; Gledhill, 1985; Kanstrup & Ekblom, 1982). A higher blood volume enhances diastolic filling of the left ventricle (Gledhill et al., 1994) resulting in a greater stroke volume because of the Frank-Starling mechanism (Coyle et al., 1990; Ekblom, 2000; Fortney et al., 1981; Kanstrup & Ekblom, 1982). It has been reported that endurance trained athletes have a higher blood volume than untrained individuals (Convertino et al., 1980; Gledhill et al., 1994; Green et al., 1990; Warburton et al., 1999), which is directly related to their enhanced aerobic performance (Krip et al., 1997; Warburton et al., 2004). In laboratory settings, acute blood volume expansion by infusion of whole blood has also been shown to improve VO_2max (Ekblom et al., 1976; Gledhill, 1985; Warburton et al., 1999), as by increasing blood volume and the number of red blood cells, more oxygen can be transported to the exercising muscles.

Plasma volume expansion independent of increases in haemoglobin and haematocrit leading to expansion of blood volume may also increase VO_2max (Coyle et al., 1990; Coyle et al., 1986; Krip et al., 1997). On the other hand, acute plasma volume expansion may also lead to haemodilution, as the number of red blood cells remains unchanged following the increase of plasma water, and this reduces the oxygen carrying capacity of the blood (Berger et al., 2006; Coyle et al., 1990; Hopper et al., 1988). However, plasma volume can be acutely expanded at an optimal level that can offset haemodilution and the improvement in stroke volume may result in positive effects on aerobic performance (Coyle et al., 1986; Coyle et al., 1990; Hopper et al., 1988; Krip et al., 1997). Despite the fact that some researchers reported no changes in VO_2max following acute plasma volume

expansion (Kanstrup & Ekblom, 1982, 1984; Mier et al., 1996), recent data from Berger et al. (2006) also showed an increase in $\text{VO}_{2\text{peak}}$ and time to exhaustion during severe-intensity exercise.

Glycerol ingestion ($1 \text{ g}\cdot\text{kg}^{-1}$ body mass in a $6 \text{ ml}\cdot\text{kg}^{-1}$ body mass solution) increases plasma volume by $\sim 8\%$ and blood volume by $\sim 5\%$ due to the osmotic properties of glycerol, as shown in the previous chapters of this thesis. This level of plasma and blood volume increase is similar to that reported to be the optimal level of expansion that results in positive effects on stroke volume and aerobic exercise (Berger et al., 2006; Coyle et al., 1986; Coyle et al., 1990; Hopper et al., 1988; Warburton et al., 1999).

The purpose of this study was to investigate potential effects of glycerol ingestion and the subsequent blood volume increase during an incremental $\text{VO}_{2\text{max}}$ cycling test.

Materials and methods

Following ethics approval by the Loughborough University Ethical Advisory Committee, eight healthy male volunteers participated in this study. After a written and oral explanation of all procedures, and their right to withdraw at any point without giving any reason for doing so, each participant signed a written consent form. All participants were physically active, but none of them was an athlete engaged in full-time training. Their age, height and body mass were, respectively, (mean \pm SD) 23 ± 3 y (range 20 – 26 y), 180 ± 7 cm, and 81.0 ± 5.9 kg. Each subject participated in two experimental trials in randomised order, which was assigned using a Latin square design.

Experimental procedure

Participants visited the laboratory on four different occasions. The first two visits were used for familiarisation with the laboratory environment and experimental

procedures, and the participants performed a VO_2max test. The other two visits were the main trials.

The two main trials were separated by at least 7 days and not more than 15 days, starting at the same time in the morning. The participants were asked to fast overnight, and follow similar activity and food intake patterns for the 24 hours preceding each trial. The participants were also asked to refrain from strenuous exercise the day before each trial. Participants kept a record of their food intake and activities for the 24 hours before each trial and stated that they had adhered to these instructions.

Participants arrived at the laboratory in the morning ($9 \text{ am} \pm 1 \text{ h}$). After voiding, body mass was measured wearing minimal clothing on a beam balance (Marsdens type 150, Marsdens Weighing Machines, London, U.K.) to the nearest 10 g. Then participants were seated comfortably in the laboratory. One arm was warmed up by immersion in hot water (42°C) for 10 min, and then, an indwelling canula (size 21 g) was inserted into a forearm vein and stayed in place throughout the session to allow multiple blood samples to be drawn. The canula was kept patent between sample collections by flushing with heparinised isotonic saline (0.9% NaCl solution).

After a 30 min rest period, participants ingested either lemon-flavoured water ($6 \text{ ml}\cdot\text{kg}^{-1}$ body mass) or a lemon-flavoured glycerol solution ($1 \text{ g}\cdot\text{kg}^{-1}$ body mass glycerol, $6 \text{ ml}\cdot\text{kg}^{-1}$ body mass volume) and remained seated for another 30 min to allow body water changes to occur. From previous work it was known that at 30 min following glycerol ingestion, plasma volume expansion was likely to be at its maximum value.

Exercise protocol

Exercise consisted of an incremental VO_2max test on an electrically braked cycle ergometer (Gould, Bilthoven, The Netherlands). The cycle ergometer seat was adjusted according to the height of each participant. Participants were allowed to pedal at a cadence of their choice, higher than 60 rpm. Starting intensity was set at 100 W, and it was increased by 50 W every 3 min until participants reached

volitional exhaustion or when they could not maintain a cadence above 60 rpm. When participants approached the last stage of exercise, the increase in intensity was adjusted according to heart rate and rate of perceived exertion, and the increase could be only 25 W instead of 50 W, if heart rate was within 10 beats of the subject's predicted maximum and/or RPE ≥ 19 . During the last minute of every stage, expired air was collected in Douglas bags before determination of oxygen and carbon dioxide content (Servomex 1400, Crawley, East Sussex, U.K.), gas volume (Harvard Dry Gas Meter, Harvard Apparatus Ltd., Kent, U.K.) and gas temperature (Edale digital thermometer).

Heart rate (HR) was monitored using a Polar heart rate monitor (Polar Team System, Polar, U.S.A.) strapped on the chest of each participant. HR was recorded immediately before exercise and at the end of each exercise stage. Rating of perceived exertion (RPE) was also recorded at the end of each stage on the cycle ergometer.

Blood sampling and analysis

Two blood samples (3 ml) were taken at **rest**, 10 min **apart**, before ingestion. Then, blood samples (3ml) were taken immediately before exercise, at the end of each stage (within the last 10 s of the stage) and immediately at the end of exercise. Blood samples were analysed for haemoglobin concentration, haematocrit, blood glucose and blood lactate concentration. Haemoglobin concentrations and haematocrit were used to estimate changes in blood, red cell and plasma volumes as described by Dill and Costill (1974). Serum samples were analysed for osmolality and glycerol concentration.

All analyses were performed as described in the general methods section of this thesis.

Statistical Analysis

A two-way ANOVA was used to find differences between trials where multiple measurements were taken. Where appropriate, a Tukey post-hoc test was used to distinguish differences between trials. One-way ANOVA was used to find

differences over time during a trial. Paired t – tests were also used where appropriate.

All data are presented as mean \pm standard deviation of the mean (SD). For all statistical analyses, significance was set at $P < 0.05$.

Results

Baseline measurements were similar during the two different experimental trials (Table 6.1).

Table 6.1 Pre-exercise body mass, Hb concentration, haematocrit, blood glucose concentration, blood lactate, serum glycerol and serum osmolality on the two trials (Mean \pm SD); n = 8.

	Water trial	Glycerol trial	P value
Body mass (kg)	79.77 \pm 5.29	79.41 \pm 5.33	0.198
Hb concentration (g·l ⁻¹)	166 \pm 17	168 \pm 17	0.311
Haematocrit (%)	44.6 \pm 1.8	44.3 \pm 2.2	0.907
Blood glucose (mmol·l ⁻¹)	5.59 \pm 0.65	5.32 \pm 0.43	0.310
Blood lactate (mmol·l ⁻¹)	0.88 \pm 0.17	0.78 \pm 0.12	0.320
Serum glycerol (mmol·l ⁻¹)	0.03 \pm 0.01	0.03 \pm 0.01	0.248
Serum osmolality (mosmol·kg ⁻¹)	286 \pm 3	288 \pm 3	0.195

Serum glycerol concentration (Table 6.2)

Two-way repeated measures ANOVA on serum glycerol showed a main effect of trial ($P = 0.000$), time ($P = 0.000$) and an interaction ($P = 0.000$). Serum glycerol increased following glycerol ingestion to $9.57 \pm 3.49 \text{ mmol}\cdot\text{l}^{-1}$ ($P = 0.000$) and remained elevated throughout the experimental trial. During the water trial, serum glycerol did not change over time and remained at baseline ($P = 0.658$).

Table 6.2 Serum glycerol concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; $n = 8$. P denotes difference between trials.

	Rest	Pre- Exercise	Stage 1	Stage 2	Stage 3	Stage 4	End Exercise
Water trial	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Glycerol trial	0.03 ± 0.01	9.57 ± 3.49	8.32 ± 2.63	8.83 ± 2.73	9.19 ± 1.70	9.66 ± 1.93	9.17 ± 1.65
P	0.178	0.000	0.000	0.000	0.000	0.000	0.000

Serum osmolality (Fig. 6.1)

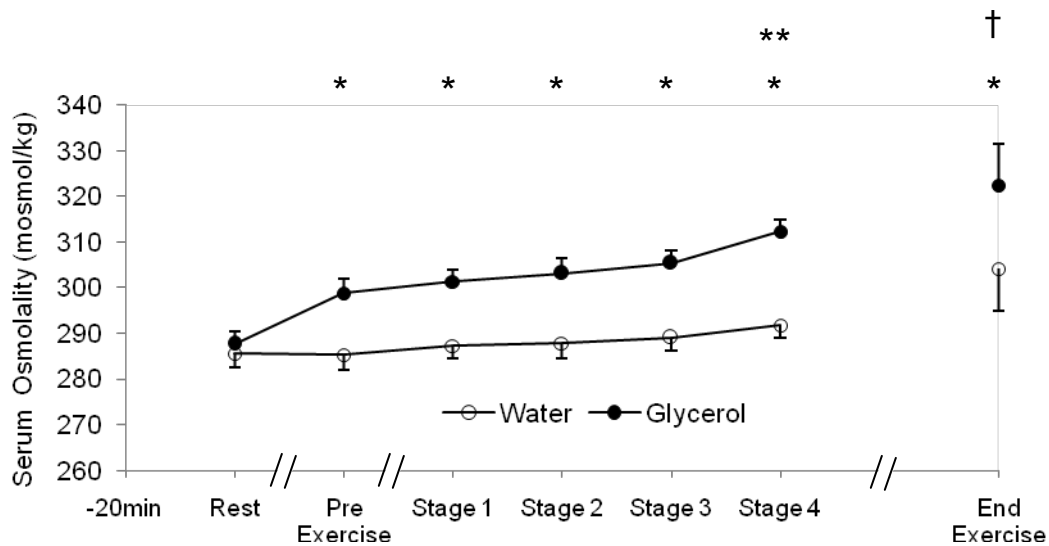
Two-factor repeated measures ANOVA on serum osmolality showed a main effect of trial ($P = 0.000$), time ($P = 0.000$) and an interaction ($P = 0.002$). Comparisons between trials showed that serum osmolality was higher on the glycerol trial at all time points following drink ingestion ($P = 0.000$) and this difference was associated with the higher serum glycerol concentration.

On the water trial, serum osmolality increased at the end of exercise to $304 \pm 9 \text{ mosmol}\cdot\text{kg}^{-1}$ and this was higher than all other time points ($P = 0.000$).

On the glycerol trial, serum osmolality increased to $299 \pm 5 \text{ mosmol}\cdot\text{kg}^{-1}$ following glycerol ingestion ($P = 0.000$) and remained higher than baseline throughout the

session. Compared with the pre-exercise point, serum osmolality increased to 312 ± 4 mosmol·kg⁻¹ at stage 4 ($P = 0.000$). At the end of exercise, serum osmolality was 322 ± 4 mosmol·kg⁻¹, and this was higher ($P < 0.001$) than all other time points in the trial.

Figure 6.1 Serum osmolality (mosmol·kg⁻¹). Points are means \pm SD; $n = 8$. * Denotes difference between trials ($P = 0.000$). ** Denotes difference from pre-exercise point on the glycerol trial. † Denotes difference from all other time points ($P = 0.000$) in both trials.

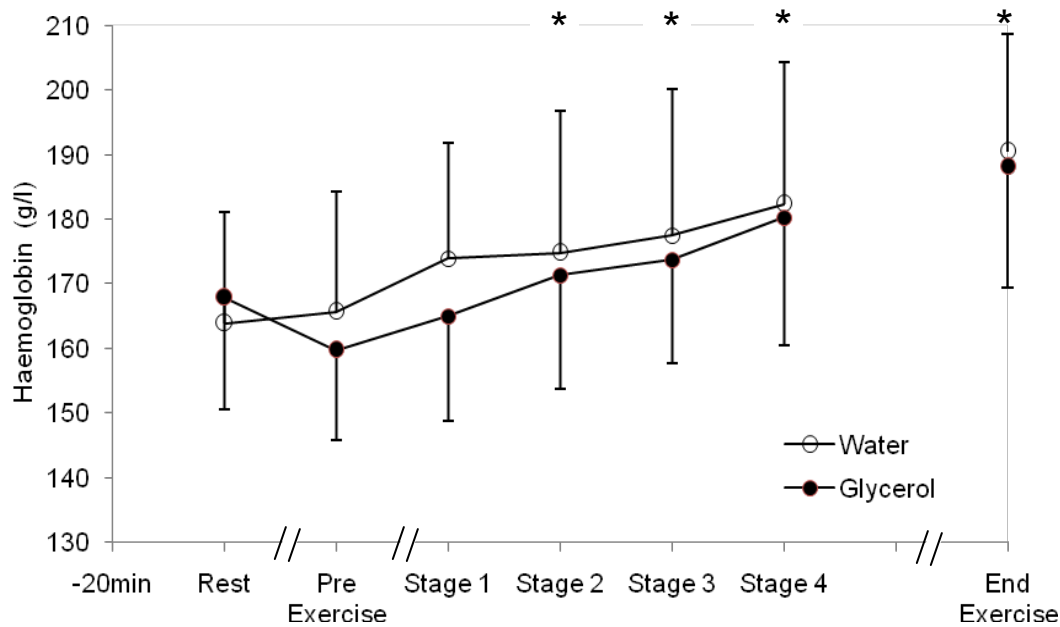


Haemoglobin concentration (Fig. 6.2)

A two-way ANOVA on Hb showed no effect of trial ($P = 0.304$), an effect of time ($P = 0.000$) and no interaction ($P = 0.062$). Hb was not different between trials ($P > 0.050$); except at the pre-exercise point, where Hb on the glycerol trial tended to be lower ($P = 0.062$) than on the water trial.

Over time comparisons for haemoglobin concentration (both trials results pooled together) showed that Hb concentration compared with the pre-exercise point (pair-wise comparisons with Tukey correction) increased during exercise reaching its maximum value at the end of exercise ($P = 0.000$).

Figure 6.2 Haemoglobin concentration (g/l). Points are means \pm SD; n = 8. * Denotes difference from the pre-exercise point for both trials together.



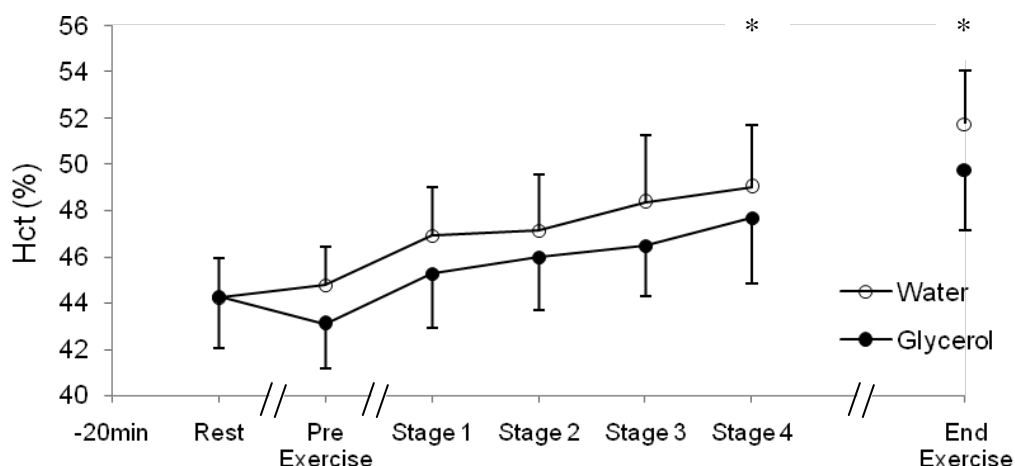
Haematocrit (Fig. 6.3)

Two-way repeated measures ANOVA on Hct showed a main effect of trial ($P = 0.002$), time ($P = 0.000$) and an interaction ($P = 0.005$).

Analysis of Hct over time showed that there was no difference between rest and the pre-exercise point (water trial $P = 0.868$, glycerol trial $P = 0.963$), but that compared with the pre-exercise point Hct increased during exercise. At stage 4, the difference reached statistical significance (water trial $P = 0.019$, glycerol trial $P = 0.012$) and Hct reached its peak value at the end of exercise (water trial $P = 0.000$, glycerol trial $P = 0.000$).

While Hct on the glycerol trial appeared to be lower than on the water trial at all time points following glycerol ingestion, comparisons between trials using an ANOVA test with pair wise comparisons indicated that there was no difference in Hct between the two trials at any time point ($P > 0.100$).

Figure 6.3 Haematocrit (%). Points are means \pm SD; n = 8. * Denotes difference over time compared with the **pre-exercise** point, on both trials.



Plasma volume changes (Fig. 6.4)

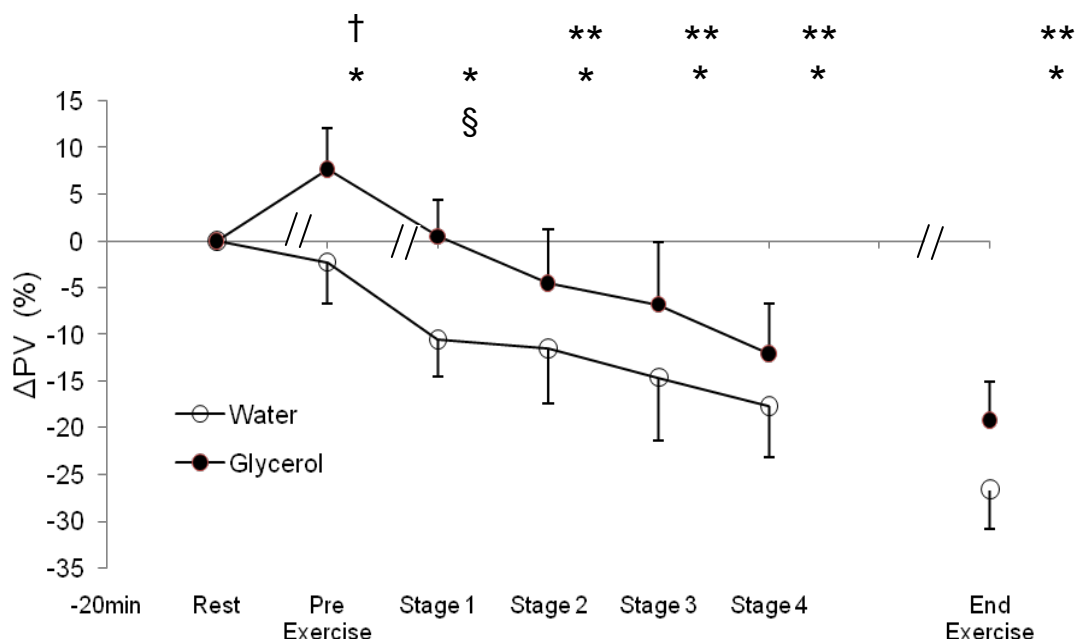
Two-way repeated measures ANOVA on plasma volume changes showed a main effect of trial ($P = 0.000$), time ($P = 0.000$) and an interaction ($P = 0.000$).

Comparisons between trials showed that plasma volume on the glycerol trial was higher at all points following glycerol ingestion ($P \leq 0.030$), except at stage 4 ($P = 0.070$).

On the water trial, plasma volume was not different between rest and pre-exercise ($P = 0.968$). During exercise, plasma volume at each stage decreased ($P < 0.050$), and at the end of exercise, it was $26.7\% \pm 4.2$ lower than baseline and statistically lower than all other time points ($P < 0.023$).

On the glycerol trial, plasma volume increased following glycerol ingestion, and at the pre-exercise point, it was $7.7\% \pm 4.6$ higher than baseline ($P = 0.034$). Plasma volume during exercise showed a statistically significant continuous decrease compared with the pre-exercise point, starting from stage 2 ($P = 0.000$). At the end of exercise, plasma volume reached its lowest value and it was $27.0\% \pm 4.1$ lower than the pre-exercise point ($P = 0.000$).

Figure 6.4 Plasma volume changes (%). Points are means \pm SD; n = 8. * Denotes difference between trials ($P \leq 0.030$). ** Denotes difference from pre-exercise point on both trials. § Denotes difference from pre-exercise point on the water trial. † Denotes difference between rest and pre-exercise point on the glycerol trial.



Blood volume changes (Fig. 6.5)

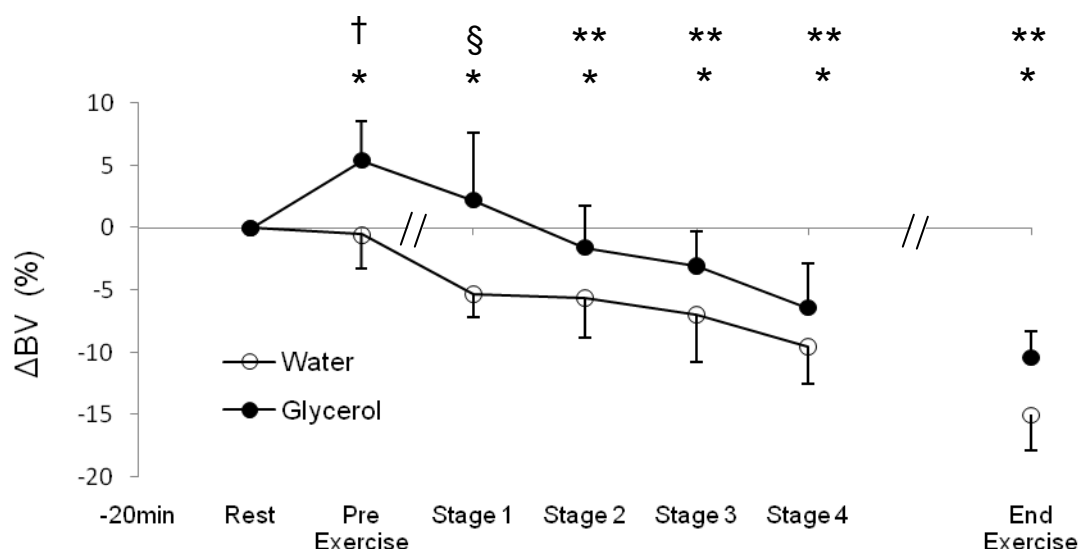
Two-way repeated measures ANOVA on blood volume showed a main effect of trial ($P = 0.001$), time ($P = 0.000$) and an interaction ($P = 0.005$).

Comparisons between trials showed that following glycerol ingestion, blood volume was higher than on the water trial at all time points ($P < 0.002$).

For the water trial, analysis over time showed that blood volume was not different between rest and pre-exercise ($P = 1.000$), but from the first stage, blood volume started to decrease ($P = 0.037$). At the end of exercise, blood volume was $14.5\% \pm 2.7$ lower than at the pre-exercise point, and this was lower than all other time points in the trial ($P \leq 0.010$).

On the glycerol trial, blood volume increased by $5.4 \% \pm 3.1$ following glycerol ingestion ($P = 0.050$). Then, blood volume decreased during exercise, reaching statistical significance from stage 2 ($P = 0.005$) onwards. At the end of exercise, blood volume was $15.8 \% \pm 2.1$ lower than at the pre-exercise point ($P = 0.000$), and statistically lower from all points ($P = 0.000$), except for that at stage 4 ($P = 0.096$).

Figure 6.5 Blood volume changes (%). Points denote means \pm SD; $n = 8$. * Denotes difference between trials. ** Denotes difference from **pre-exercise** point on both trials. § Denotes difference from pre-exercise point on the water trial. † Denotes difference between rest and **pre-exercise** point on the glycerol trial.



Red cell volume (Table 6.3)

Two-way repeated measures ANOVA on red cell volume showed no effect of time ($P = 0.260$), no trial effect ($P = 0.517$) and no interaction ($P = 0.341$).

Table 6.3 Red cell volume (%). Values are presented as means \pm SD; n = 8. P denotes difference between trials.

	Rest	Pre - exercise	Stage 1	Stage 2	Stage 3	Stage 4	End Exercise
Water	44.2	45.0	44.8	45.1	45.4	44.5	44.1
trial	± 1.7	± 1.3	± 1.9	± 1.5	± 1.7	± 1.5	± 1.5
Glycerol	44.3	45.7	46.3	45.2	45.0	44.6	44.6
trial	± 2.2	± 2.7	± 3.5	± 2.3	± 2.5	± 2.4	± 2.5
P	0.897	0.696	0.224	0.880	0.626	0.956	0.353

VO₂max test parameters (Table 6.4)

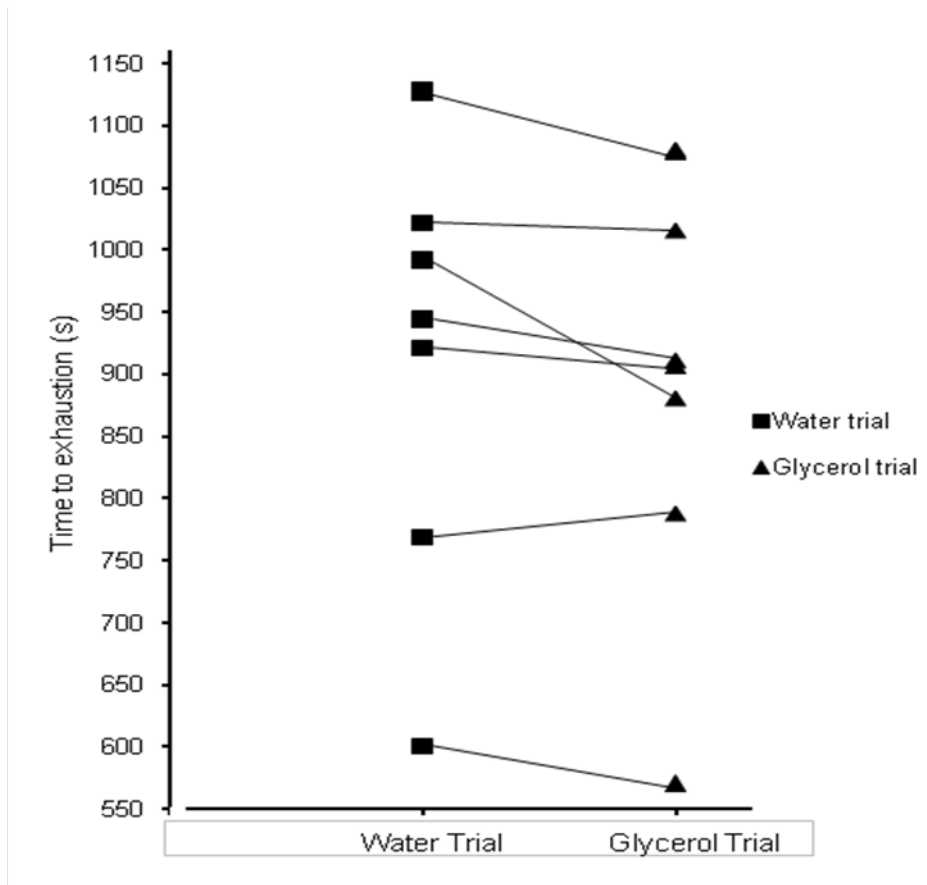
Paired t – test analyses showed that there was no difference between the two trials in the maximum workload reached at the end of exercise (P = 0.180), the VO₂max attained (P = 0.770), the maximum heart rate (P = 0.100) or the respiratory exchange ratio (RER) at maximum workload (P = 0.700).

Paired t – test analysis on time to fatigue showed that participants reached fatigue slightly sooner on the glycerol trial (P = 0.044). All participants exercised for slightly less on the glycerol trial, except for only one subject (Figure 6.6).

Table 6.4 VO₂max test parameters. Values are presented as means \pm SD; n = 8. * Denotes significant difference between the two experimental trials (P < 0.05).

	Water Trial	Glycerol Trial
VO ₂ max (ml·kg ⁻¹ ·min ⁻¹)	51.2 \pm 8.1	50.5 \pm 10.4
Workload (W)	313 \pm 44	300 \pm 59
HR (bpm)	185 \pm 8	178 \pm 12
RER	1.14 \pm 0.08	1.13 \pm 0.05
Time to fatigue (s)	937 \pm 179	904 \pm 167 *

Figure 6.6 Individual cycling exercise time (s) for all subjects (n = 8).



Blood lactate concentration (Table 6.5)

Two-way repeated measures ANOVA of blood lactate data showed a time effect ($P = 0.000$), but no trial effect ($P = 0.995$) and no interaction ($P = 0.811$). Pair-wise comparisons with a Tukey adjustment showed that compared with pre-exercise measurements, lactate concentration were greater at stage 4 ($P < 0.003$), and had increased further by the end of exercise ($P = 0.000$).

Table 6.5 Blood lactate concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; n = 8. * Denotes difference from pre-exercise point, stages 1 & 2 ($P < 0.020$) for the water trial. ** Denotes difference from all previous time points ($P = 0.000$)

	Rest	Pre Exercise	Stage 1	Stage 2	Stage 3	Stage 4	End Exercise
Water	0.88	1.18	1.52	1.85	2.81	5.15*	13.31**
trial	± 0.77	± 0.28	± 0.58	± 0.55	± 1.10	± 2.64	± 3.55
Glycerol	0.78	1.33	1.61	1.85	2.71	5.51**	12.91**
trial	± 0.12	± 0.16	± 0.55	± 0.51	± 0.89	± 1.69	± 3.50

Blood glucose concentration (Table 6.6)

Two-way repeated measures ANOVA of blood glucose data showed a main time effect ($P = 0.035$), but no effect of trial ($P = 0.687$), and no interaction ($P = 0.338$), and that blood glucose was lower at stage 2 ($P = 0.001$) and stage 3 ($P = 0.035$) compared with the end of exercise point. One-way ANOVA over time within trials showed no significant change in blood glucose concentration on either trial (water trial $P = 0.695$, glycerol trial $P = 0.951$).

Table 6.6 Blood glucose concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; n = 8. P denotes difference between trials.

	Rest	Pre Exercise	Stage 1	Stage 2	Stage 3	Stage 4	End Exercise
Water	5.50	5.37	5.16	5.27	5.35	5.51	5.68
trial	± 0.64	± 0.53	± 0.59	± 0.60	± 0.53	± 0.46	± 0.57
Glycerol	5.32	5.39	5.31	5.12	5.19	5.37	5.57
trial	± 0.43	± 0.64	± 1.04	± 0.68	± 0.62	± 0.76	± 0.83
P	0.554	0.947	0.755	0.679	0.625	0.686	0.778

Discussion

The aim of this study was to investigate potential effects of glycerol ingestion and subsequent blood volume expansion during an incremental cycling VO_2max test. The hypothesis was that increased blood volume would lead to positive effects on aerobic performance during the cycling test. However, even although blood volume was increased, VO_2max remained unchanged, and time to exhaustion was reduced on the glycerol trial.

The importance of blood volume for aerobic cardiovascular capacity has been well studied and documented. Blood volume increases following endurance training (Convertino et al., 1980; Green et al., 1990; Gillen et al., 1991) and endurance trained individuals have a greater blood volume and exhibit greater stroke volume and cardiac output than untrained individuals during maximal exercise (Ekblom & Hermansen, 1968). For untrained individuals, acute expansion of blood volume using a blood volume expander (i.e. dextran) has been reported to result in an immediate, considerable effect on diastolic filling time and rate, and thus on maximum stroke volume and cardiac output (Coyle et al., 1990; Kanstrup & Ekblom, 1982; Krip et al., 1997). Other investigators (Kanstrup & Ekblom, 1984; Mier et al., 1996; Warburton et al., 1999) failed to find any increases in VO_2max following plasma volume expansion, but plasma expansion in these studies was 500 – 700 ml. This greater degree of plasma volume expansion resulted in a significant decrease in haemoglobin concentration ($> 8\%$), reducing the oxygen carrying capacity per unit volume of blood. VO_2max was not reduced in these studies because the increase in cardiac output following blood volume expansion appeared to compensate for the haemodilution and there was no decrease in aerobic exercise performance despite the reduced oxygen carrying capacity of the blood.

In the present study, glycerol ingestion resulted in a 5.4% increase in blood volume and a 7.7% increase in plasma volume due to the osmotic properties of glycerol. Haemoglobin was not significantly reduced following glycerol ingestion, thus haemodilution did not appear to be an issue. The change in plasma volume

was estimated to be ~ 300 ml (Guyton & Hall, 1996). Coyle et al. (1990) and Hopper et al. (1988) suggested that a plasma volume expansion of 400 ml or less in untrained participants seems to be the optimum level for increasing stroke volume and cardiac output. Coyle et al. (1990) reported that 200 - 300 ml of dextran infusion resulted in a 10 – 15% increase in stroke volume and cardiac output allowing VO_2max to increase by 4%. Berger et al. (2006) reported that infusion of 500 ml of 6% dextran improved maximum stroke volume by 9.1% and maximum cardiac output by 8.9%, resulting in an increased VO_2max by 7%. In the present study, despite what seemed to be a beneficial blood volume expansion, VO_2max did not increase following glycerol ingestion.

The major factor leading to fatigue during an incremental cycling VO_2max test has been shown to be the impaired O_2 supply due to a fall in stroke volume as arterial O_2 content, heart rate and O_2 extraction increase during exercise (Mortensen et al., 2005). A larger blood volume may help maintain adequate filling pressure and end diastolic volume at high heart rates where heart diastole time is shorter (Gledhill et al., 1994). Any difference in either heart rate or blood volume during exercise may affect stroke volume and VO_2 . In the present study, heart rate was similar on the two trials; however, blood volume was different. Participants on the glycerol trial started exercise with a higher blood volume. During exercise, blood volume decreased on both trials and this was attributed to a decrease in plasma volume as there was no change in red cell volume at any time point on any of the two trials. Plasma volume decreased almost linearly as exercise intensity and muscle power increased. The exit of water from the vascular space is largely attributed to the accumulation of lactate in the muscle, which has been shown to be double the concentration of blood lactate (Sjogaard & Saltin, 1982), thus creating an osmotic gradient and entry of water into the muscle. The decrease in plasma volume was similar in both trials (~27%) compared with the pre-exercise point. This is in agreement with the decrease in plasma volume (21 – 27%) reported by Sjogaard and Saltin (1982) following a short cycling session at 120% VO_2max to exhaustion that resulted in a 15% increase in intramuscular water of the exercising muscles which, according to the authors, explained the loss of

water from the plasma. Sjogaard et al. (1985) reported a 21% increase in total muscle water following single leg dynamic knee extension to fatigue at 100% VO_2max of the knee extensors. The loss of water from the blood also leads to haemoconcentration. A haemoconcentration from rest to maximal exercise of between 10 – 15% is regarded as normal (Ekblom & Hermansen, 1968). In the present study, haemoconcentration from rest to the end of exercise was 15% on the water trial and 12% on the glycerol trial. The results of the present study show that as participants on the glycerol trial initiated exercise in an elevated blood volume state the decrease in blood and plasma volume during exercise was similar in the two trials, but blood and plasma volume were higher on the glycerol trial at the point of exhaustion. Based on the fact that a higher blood volume enhances stroke volume and is beneficial for aerobic exercise, participants on the glycerol trial should benefit from the higher blood volume, but this was not the case in the present study, as the participants reached exhaustion slightly sooner. One major difference between the present study and other studies investigating plasma volume expansion is that participants in other studies were well hydrated and more fluid was infused to further increase plasma volume. In the present study, participants ingested glycerol, which resulted in an increased serum osmolality. Ingestion of highly hypertonic fluids may result in altered body water distribution (see chapter 3 of this thesis) and the subsequent increase in plasma volume could possibly be at the expense of cell volume and cell hydration (Hamilton et al., 1993) which, in effect, may increase exercise stress (Judelson et al., 2007). It is possible that the change in body water distribution on the glycerol trial impaired skeletal muscle aerobic energy provision and work capacity during maximal exercise, thus reversing the effects of increased stroke volume during high intensity aerobic exercise.

Energy during cycling at high intensity derives from glycogen breakdown and the main product is lactic acid, which is completely ionized to lactate and free hydrogen ions (H^+) (Maughan et al., 1997). Blood lactate measured in the present study was not different between trials at the point of exhaustion, but exhaustion on the glycerol trial occurred sooner, possibly indicating an accelerated

glycogenolysis and lactate production or release into the blood. On the water trial, blood lactate significantly increased from baseline at stage 4 and only at the end of exercise was blood lactate higher than at all of the previous stages. On the glycerol trial, blood lactate increased from baseline more abruptly, and, at stage 4, it was higher than at all the previous time points on the trial. Exposure of cells to a hypertonic environment and the ensuing cell shrinkage has also been shown to increase lactate production (Antolic et al., 2007; Lang et al., 1989). Increased lactate, which is accompanied by an increase in H^+ concentration and the ensuing muscle acidosis are linked with a decline in development of muscle tension (Bangsbo et al., 1996). In vitro studies have shown that a fall in pH has a direct depressant effect on the contractile machinery. Fabiato and Fabiato (1978) and Godt and Nosek (1989) showed that a decreasing pH results in decreased Ca^{2+} sensitivity of skinned skeletal muscles and a decrease of the maximum tension obtained in the presence of an optimal free Ca^{2+} concentration. It is possible that an abrupt rise in H^+ , as mirrored by the lactate accumulation observed on the glycerol trial, contributed to the earlier development of fatigue.

Another factor also related to muscle contractions and fatigue is muscle potassium concentration. Excessive potassium efflux and increased extracellular potassium may lead to reduced muscle fibre excitability and reduced ability to respond to motor signals (Nielsen & de Paoli, 2007) leading to fatigue. Potassium is lost from muscle during intense exercise (Bangsbo et al., 1996; Vollestad et al., 1994). Potassium efflux may also increase due to increases in H^+ (Davies, 1990; Usher-Smith et al., 2009). Cell shrinkage (reduced cellular hydration) is also accompanied by potassium efflux through the opening of potassium channels and intracellular potassium decreases (Haussinger et al., 2004). It is possible that on the glycerol trial there was an increased loss of potassium from the muscle. However, as electrolytes were not measured in the present study it is impossible to confirm this speculation.

The significant finding of this study is that despite the increase in blood volume following glycerol ingestion, maximum oxygen uptake (VO_{2max}) was not improved and that time to exhaustion was reduced despite the fact that the maximum

workload achieved was similar on the two trials. This negative outcome may possibly be related to the hyper-osmotic conditions that the muscles were exposed to following glycerol ingestion, and muscle function might have been compromised. From the data obtained from this study, it was not possible to reach any definite conclusions relating to the mechanism leading to fatigue. Since the only difference between the two trials occurred at the last stage of the VO_2max test, the next area of focus would be to investigate aerobic performance at high intensity 100% VO_2max . Apart from lactate measured in the present study, it would be helpful to monitor electrolyte concentration, such as potassium, which may play a role in muscle function and fatigue development.

Chapter 7

Effects of glycerol ingestion on exercise capacity at 100% VO₂max intensity

Introduction

Results from the study described in the previous chapter of this thesis showed that blood volume expansion following ingestion of a glycerol solution ($1\text{g}\cdot\text{kg}^{-1}$ body mass; $6\text{ ml}\cdot\text{kg}^{-1}$ body mass) did not improve VO_2max during an incremental cycling protocol, and that time to exhaustion was decreased on the glycerol trial.

In the literature, increases in blood volume as a result of endurance training are associated with improvements in VO_2max , endurance and enhancement in aerobic exercise performance (Convertino., 1991). This is mainly due to an improved stroke volume and cardiac output caused by the Frank–Starling mechanism (Coyle et al., 1990; Gledhill et al., 1999). In untrained individuals, induced plasma volume expansion with dextran has also been shown to improve maximal oxygen uptake (Berger et al., 2006; Coyle et al., 1990; Coyle et al., 1986; Krip et al., 1997), which can lead to improved exercise performance. Coyle et al. (1986) showed an increase in time to fatigue during upright cycling following plasma volume expansion with dextran. Luetkemeier and Thomas (1994) showed that induced hypervolaemia had a beneficial effect on performance and average power output during a cycling time trial protocol. More recently, Berger et al. (2006) reported that acute hypervolaemia increased time to exhaustion during cycling at high intensity against a constant workload.

On the other hand, Kanstrup and Ekblom (1982) observed no change in VO_2max following acute plasma volume expansion with an average of 700 ml of a dextran solution and Green et al. (1987) also reported no change in VO_2max following a 624 ml increase in plasma volume after short-term training. Coyle et al. (1990) reported that a modest plasma volume expansion (7.3%) in untrained individuals was associated with a significant increase in VO_2max , whereas a larger plasma volume expansion (17%) resulted in no change in this parameter. They suggested that there may be an optimal increase in plasma volume (200 – 300 ml), where the increase in stroke volume is not offset by a proportional dilution in haemoglobin concentration. As indicated in chapter 6 of this thesis, increases in plasma volume (7.7%) following ingestion of a glycerol solution (glycerol $1\text{g}\cdot\text{kg}^{-1}$ body mass; total

solution volume $6 \text{ ml} \cdot \text{kg}^{-1}$ body mass) were similar to those reported by Coyle et al. (1990), as well as those reported by Luetkemeier and Thomas (1994) that reported improved maximal oxygen uptake and improved exercise performance during a simulated cycling time trial.

In the previous chapter of this thesis, participants were untrained and it was hypothesized that an improvement could occur on the glycerol trial both in oxygen uptake and time to fatigue. While VO_2max was similar in the two trials, there was a slight decrease in time to exhaustion when participants were cycling at 100% VO_2max intensity on the glycerol trial. The amount of blood and plasma volume expansion was moderate and haemoglobin dilution was not an issue in this study. Since there was no difference in any of the measured variables (VO_2 , RER, heart rate, blood lactate) in the submaximal intensities, and the only difference occurred at the last stage of the incremental cycling test, it would be of interest to further examine the effects of glycerol ingestion at 100% VO_2max intensity.

Another factor to consider was that ingestion of glycerol also results in an increase in serum osmolality that is likely to lead to a water shift from intracellular to the extracellular space (Antolic et al., 2007; Blinks et al., 1965; Haussinger et al., 1994, Lang et al., 1989; Low et al., 1996), resulting in tissue dehydration (Gleeson et al., 1986; Graf & Haussinger, 1996). This, in effect, may lead to increased lactate production (Antolic et al., 2007; Lang et al., 1989), which is accompanied by hydrogen ions accumulation and is a fatiguing factor. The movement of water may also affect ionic balance which, in turn, could affect performance, especially at high intensity, where ionic regulation and fatigue seem to be linked (Cairns & Lindinger, 2008).

The aim of this study was to investigate potential effects of glycerol ingestion and the subsequent body water alteration and blood volume increase on time to fatigue during a single cycling bout at 100% VO_2max intensity.

Materials and methods

Following ethical approval by the Loughborough University Ethical Advisory Committee, eight healthy male volunteers participated in this study. After a written and oral explanation of all procedures and their right to withdraw at any point without giving any reason for doing so, each participant signed a written consent form. All participants were physically active, but none of them was an athlete engaged in serious physical training; they had a VO_2max of $45.6 \pm 8.4 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ (range $37.8 - 59.3 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). Their age, height and body mass were, respectively, (mean \pm SD) $27 \pm 2 \text{ y}$, $179 \pm 9 \text{ cm}$, and $79.6 \pm 12.1 \text{ kg}$. Each subject participated in two experimental trials in randomised order, which was assigned using a Latin square design.

Experimental procedure

Participants visited the laboratory on four different occasions. The first visit was used to measure VO_2max , the second visit was used for familiarisation with the main exercise protocol and the other two visits were when the two main trials were carried out. VO_2max was assessed using an intermittent protocol, as described in the general methods of this thesis. VO_2max was indicated by the achievement of the objective criteria of $\text{RER} > 1.1$ and a peak heart rate at least equal to 90% of the age-predicted maximal (Caputo & Denadai, 2008; Duncan et al., 1997; Taylor et al., 1955). Additionally, all subjects expressed $\text{RPE} = 20$ during the last stage of the incremental cycling test. The results of the VO_2max test and the maximum workload achieved was used to determine the absolute workload to be used during the main experimental trials. During the second visit, participants completed the 'cycling to exhaustion at 100% VO_2max intensity protocol' that they would undertake in the main experimental trials. However, the blood sampling protocol was not included in the familiarisation session.

Data was gathered from each participant in the two experimental trials that occurred in randomised order, separated by a period of seven days. Trials began

at the same time in the morning and participants followed similar physical activity and nutritional patterns for 24 hours prior to the start of each trial.

After an overnight fast and upon arrival at the laboratory, participants voided and body mass was measured (with minimal clothing on participants) on a beam balance (Marsdens type 150, Marsdens Weighing Machines, London, U.K.) to the nearest 10 g. Then participants were comfortably seated in the laboratory. One arm was warmed up by immersion in hot water (42°C) for 10 min, and then a canula (butterfly, 21 g) was inserted into a superficial forearm vein. The canula remained in place for the duration of the trial and it was kept patent between sample collections by flushing with heparinised isotonic saline (0.9 %). While participants were at rest, blood samples of 3 ml each were taken from each participant, 10 min apart. Then participants were given 3 min to ingest either 6 ml·kg⁻¹ body mass lemon-flavoured water or an equal volume of a lemon-flavoured glycerol solution (1 g·kg⁻¹ body mass; total volume of solution 6 ml·kg⁻¹ body mass). Participants remained seated for the duration of the resting period to avoid the previously reported postural changes in plasma volume (Hagen et al., 1978; Shirreffs & Maughan, 1994). Another pre-exercise 3 ml blood sample was taken 25 min after ingestion while participants were still at rest. The participant then moved to the cycle ergometer.

Exercise was performed on an electrically braked cycle ergometer (Gould, Biliithoven, The Netherlands). The cycle ergometer seat was adjusted at the appropriate height for each participant and the same seat height was used for both trials. Exercise commenced 30 min after glycerol ingestion because from previous work (as described in chapter 3 of this thesis), it was known that following glycerol ingestion, expansion of plasma and blood volume peaks at ~30 min. Cycling workload for both experimental trials was set at the maximum workload achieved during the preliminary VO₂max test for that individual. Exercise started with 5 min cycling at a workload corresponding to 50% VO₂max (low intensity warm up). A 4 ml blood sample was taken at the end of this stage and immediately after the exercise intensity was increased to elicit the subject's 100% VO₂max (high intensity). The workload remained constant and participants were asked to cycle

at this intensity for as long as they could. Exercise was terminated when the participant could not maintain a cadence above 60 rpm. Throughout exercise, participants were allowed to pedal at a cadence of their choice, but higher than 60 rpm. Participants were given strong verbal encouragement throughout the high-intensity exercise stage and the time to exhaustion was recorded to the nearest second. A final 3 ml blood sample was taken immediately after cessation of exercise.

Heart rate was monitored using a Polar heart rate monitor (Polar Team System, Polar, U.S.A.) and recorded immediately before exercise, at the end of the warm-up phase and at the end of exercise. Rating of perceived exertion (RPE) was also recorded at the end of the warm-up phase and at the end of exercise.

Blood sample analysis

Blood samples were analysed for haemoglobin (Hb) concentration, haematocrit (Hct), blood glucose concentration and blood lactate concentration. Haemoglobin concentrations and haematocrit were used to estimate changes in blood, red cell and plasma volumes, as described by Dill and Costill (1974).

Serum samples were analysed for osmolality, glycerol concentration, sodium concentration and potassium concentration.

All analyses were performed as described in the general methods section of this thesis.

Statistical analysis

A two-way ANOVA was used to find differences between trials where multiple measurements were taken. Where appropriate, a Tukey post-hoc test was used to distinguish differences between trials. One-way ANOVA was used to find differences over time during a trial. Paired t – tests were also used where appropriate.

All data are presented as mean \pm standard deviation of the mean (SD). For all statistical analyses, significance was set at $P < 0.05$.

Results

Baseline measurements were similar during the two different experimental trials (Table 7.1).

Table 7.1 Pre - exercise body mass, haemoglobin concentration, haematocrit, blood glucose concentration blood lactate, serum osmolality, serum glycerol, sodium and potassium on the two trials (Mean \pm SD); n = 8.

	Water Trial	Glycerol Trial	P
Body mass (kg)	78.8 \pm 12.4	78.6 \pm 12.4	0.220
Hb (g·l ⁻¹)	166 \pm 16	167 \pm 18	0.929
Hct (%)	45.1 \pm 2.5	44.5 \pm 2.7	0.110
Blood glucose (mmol·l ⁻¹)	4.62 \pm 0.20	4.53 \pm 0.25	0.534
Blood lactate (mmo·l ⁻¹)	0.7 \pm 0.3	0.6 \pm 0.3	0.290
Serum osmolality (mosmol·kg ⁻¹)	281 \pm 4	282 \pm 3	0.677
Serum glycerol (mmol·l ⁻¹)	0.04 \pm 0.01	0.03 \pm 0.01	0.256
Serum sodium (mmo·l ⁻¹)	142 \pm 1	142 \pm 1	1.000
Serum potassium (mmol·l ⁻¹)	4.7 \pm 0.4	4.5 \pm 0.1	0.217

Serum glycerol concentration (Table 7.2)

Two-way repeated measures ANOVA on serum glycerol concentration showed that there was a main drink effect (P = 0.000), a time effect (P = 0.000) and an interaction (P = 0.000).

On the water trial, one-way ANOVA analysis over time showed that compared with rest, serum glycerol concentration did not change over time (P = 0.172).

On the glycerol trial, one-way ANOVA analysis over time showed that serum glycerol concentration increased following glycerol ingestion (P = 0.000). From the rest concentration of 0.03 \pm 0.01 mmo·l⁻¹, serum glycerol concentration increased to 7.07 \pm 1.55 mmo·l⁻¹ at the pre-exercise point. During exercise, serum glycerol concentration tended to decrease compared with the pre-exercise point.

At the end of the low-intensity stage, serum glycerol concentration was 5.70 ± 1.04 $\text{mmo}\cdot\text{l}^{-1}$ ($P = 0.058$), and at the end of exercise, 5.72 ± 0.90 $\text{mmo}\cdot\text{l}^{-1}$ ($P = 0.063$). Comparison between trials showed that following drink ingestion, serum glycerol concentration was higher on the glycerol trial at all time points ($P = 0.000$).

Table 7.2 Serum glycerol concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; $n = 8$. P denotes statistical difference between trials.

	Rest	Pre-exercise	Low intensity	High intensity
Water trial	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
Glycerol trial	0.03 ± 0.01	7.07 ± 1.55	5.70 ± 1.04	5.72 ± 0.90
P between trials	0.255	0.000	0.000	0.000

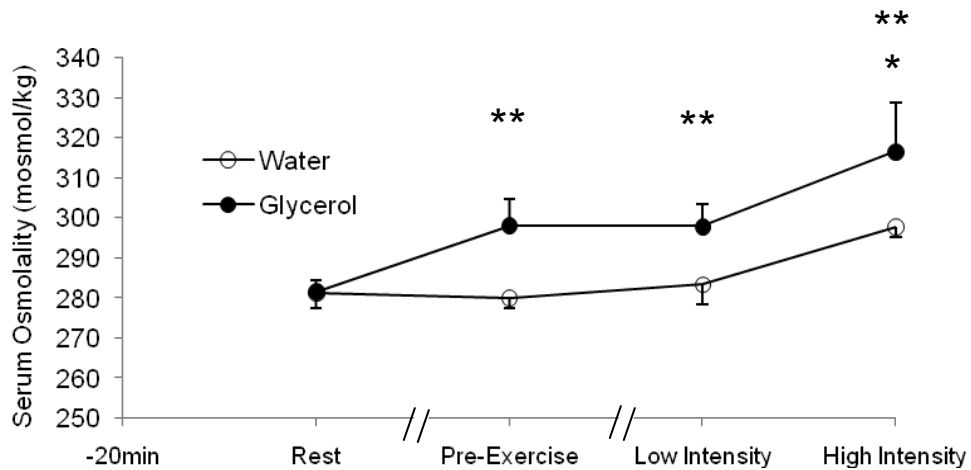
Serum osmolality (Figure 7.1)

Two-way repeated measures ANOVA on serum osmolality showed a main drink effect ($P = 0.000$), time effect ($P = 0.000$) and an interaction ($P = 0.001$).

On the water trial, one-way ANOVA analysis over time showed that serum osmolality increased at the end of exercise (high intensity) to 298 ± 2 $\text{mosmol}\cdot\text{kg}^{-1}$ and this was higher than all other time points on that trial.

On the glycerol trial, one-way ANOVA analysis over time showed that serum osmolality following glycerol ingestion at the pre-exercise point (298 ± 7 $\text{mosmol}\cdot\text{kg}^{-1}$) was higher than the rest point ($P = 0.001$). Serum osmolality at high intensity increased further ($P = 0.000$) to 317 ± 12 $\text{mosmol}\cdot\text{kg}^{-1}$ and this was higher than all other time points on the glycerol trial ($P = 0.000$).

Figure 7.1 Serum osmolality ($\text{mosmol}\cdot\text{kg}^{-1}$). Points denote means \pm SD; $n = 8$. * Denotes difference compared with all other time points within trials. ** Denotes difference between trials. ($P < 0.050$)



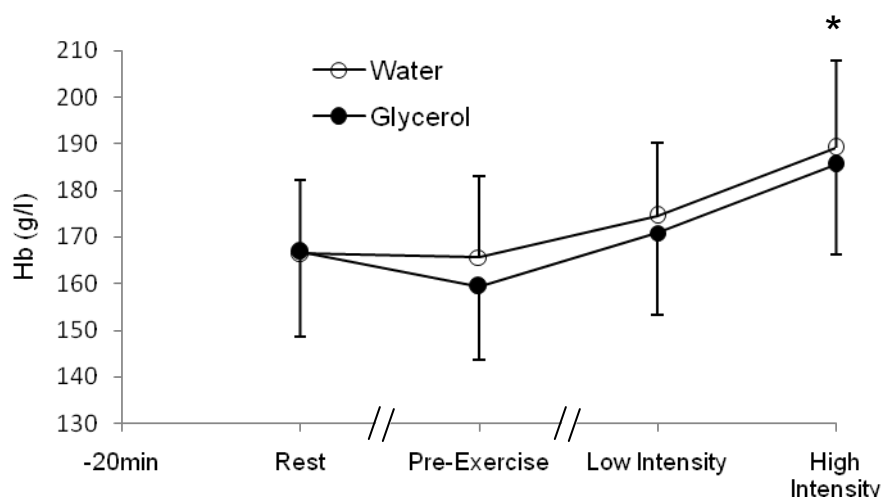
Haemoglobin concentration (Fig. 7.2)

Two-way repeated measures ANOVA on haemoglobin concentration showed a main effect of time ($P = 0.000$), no effect of trial ($P = 0.250$) and an interaction ($P = 0.006$).

On the water trial, one-way ANOVA over time with pair-wise comparisons showed that Hb concentration at 100% VO_2max at the end of exercise ($189 \pm 19 \text{ g}\cdot\text{l}^{-1}$) was higher compared with the pre-exercise point ($P = 0.042$).

On the glycerol trial, one-way ANOVA over time with pair-wise comparisons showed that Hb increased at 100% VO_2max intensity and it was higher compared with the pre-exercise point value ($P = 0.031$).

Figure 7.2 Haemoglobin concentration ($\text{g}\cdot\text{l}^{-1}$). Points are means \pm SD; $n = 8$. * Denotes difference from the pre – exercise point on both trials. $P < 0.050$.



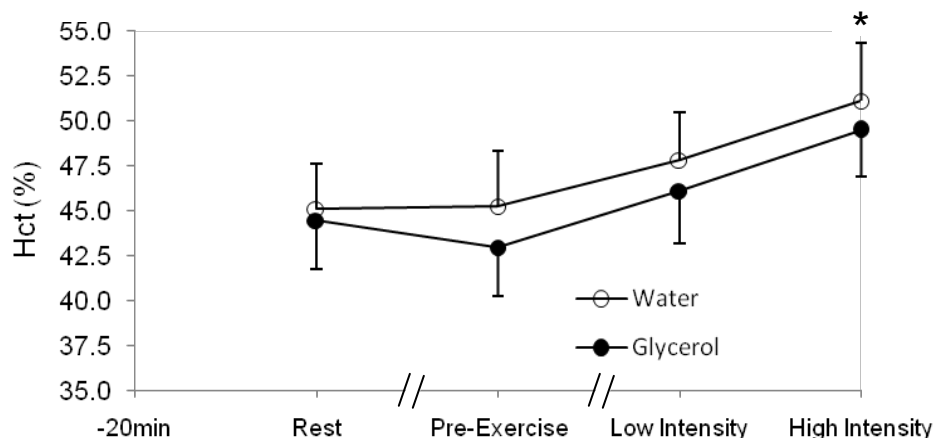
Haematocrit (Fig. 7.3)

Two-factor repeated measures ANOVA on Hct showed a main trial effect ($P = 0.000$), a main time effect ($P = 0.011$) and an interaction ($P = 0.027$). Simple pair t-tests between trials at corresponding time points showed that Hct was not different at rest ($P = 0.110$), following glycerol ingestion, Hct on the glycerol trial was lower than on the water trial at the pre-exercise point ($P = 0.005$), at 50% VO_2max intensity ($P = 0.032$) and at 100% VO_2max intensity ($P = 0.013$), but pair-wise comparisons between trials using an ANOVA test showed that there were no real differences between trials at any time point.

On the water trial, one-way ANOVA analysis over time with pair-wise comparisons (Tukey) showed that Hct increased at high intensity ($51.1\% \pm 3.3$) and this was higher than the pre-exercise value ($P = 0.002$).

On the glycerol trial, one way ANOVA analysis over time with pair wise comparisons (Tukey) showed that Hct increased to $49.5\% \pm 2.5$ at the high intensity and this was higher than the pre – exercise point ($P = 0.000$).

Figure 7.3 Haematocrit (%). Points are means \pm SD; n = 8. * Denotes difference from the **pre-exercise** point on both trials. $P < 0.050$.



Plasma volume changes (Fig. 7.4)

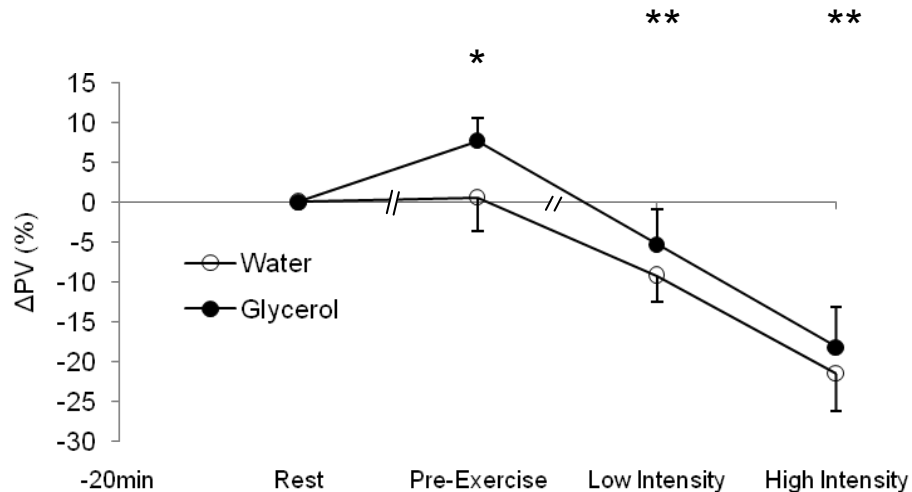
Two-way repeated measures ANOVA on plasma volume changes showed a main trial effect ($P = 0.003$), a time effect ($P = 0.000$) and an interaction ($P = 0.006$). Comparisons between trials (ANOVA test) showed that plasma volume on the glycerol trial was higher than on the water trial at only the pre-exercise point ($P = 0.001$).

On the water trial, one-way ANOVA with pair-wise comparisons showed that plasma volume was not different between rest and the pre-exercise point ($P = 0.991$). During exercise, plasma volume had decreased by $9.2\% \pm 2.9$ at the end of the low-intensity exercise ($P = 0.000$) and at the end of the high intensity exercise by $21.5\% \pm 4.8$ compared with the pre-exercise ($P = 0.000$). Plasma volume values were also statistically different between the two exercise intensities ($P = 0.000$).

On the glycerol trial, one-way ANOVA with pair-wise comparisons showed that following glycerol ingestion, plasma volume increased by $7.7\% \pm 2.9$ at the pre-exercise point ($P = 0.002$). During exercise, plasma volume decreased compared with the pre-exercise point by $13.0\% \pm 4$ at the end of the low intensity exercise (P

= 0.000), and by $25.9\% \pm 3.9$ at the end of the high intensity exercise ($P = 0.000$). Plasma volume values were also statistically different between the two exercise intensities ($P = 0.000$).

Figure 7.4 Plasma volume changes (%). Points denote means \pm SD; $n = 8$. * Denotes difference between trials. ** Denotes difference compared with the pre-exercise point and between exercise intensities ($P \leq 0.002$) on both trials.



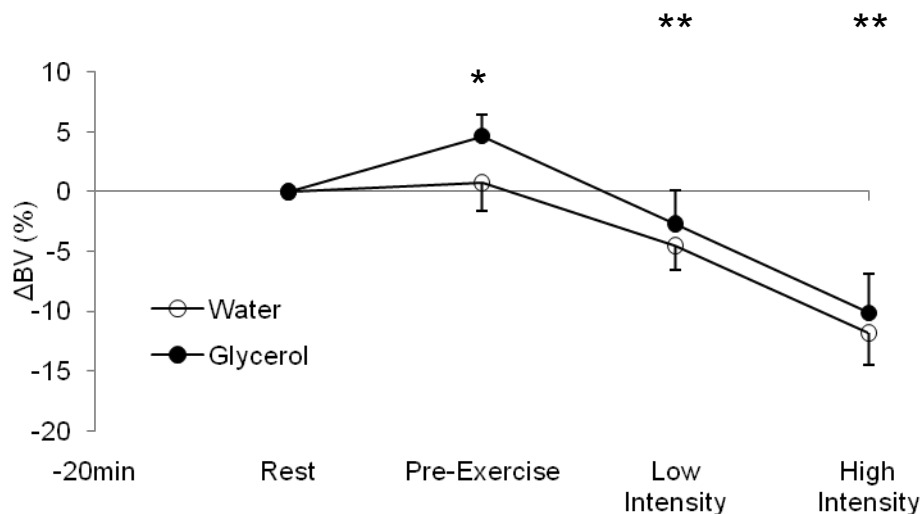
Blood volume changes (Fig. 7.5)

Two-way repeated measures ANOVA on blood volume changes showed a main trial effect ($P = 0.004$), a time effect ($P = 0.000$) and an interaction ($P = 0.010$).

On the water trial, one-way ANOVA analysis with pair-wise comparisons showed that blood volume between rest and pre-exercise point was not different ($P = 0.875$). Compared with the pre-exercise point, during exercise, blood volume decreased by $5.3\% \pm 2.5$ ($P = 0.000$) at the end of the low-intensity stage and by $12.6\% \pm 2.5$ ($P = 0.000$) at the end of the high intensity exercise. Blood volume between exercise intensities was also statistically different ($P = 0.000$).

On the glycerol trial, one-way ANOVA analysis with pair-wise comparisons showed that the $4.7\% \pm 1.8$ increase in blood volume at the pre-exercise point following glycerol ingestion was statistically higher than the rest point ($P = 0.002$). During exercise, blood volume decreased compared with the pre-exercise point ($P = 0.000$) by $7.3\% \pm 2.4$ at the end of the low-intensity stage ($P = 0.000$) and by $14.8\% \pm 2.7$ at the end of the high intensity ($P = 0.000$). Blood volume values between exercise intensities were also statistically different ($P = 0.000$). Comparisons between trials (using ANOVA test with pairwise comparisons) showed that blood volume was higher on the glycerol trial than on the water trial at only the pre-exercise point ($P = 0.003$).

Figure 7.5 Blood volume changes (%). Points are means \pm SD; $n = 8$. * Denotes difference between trials ($P < 0.050$). **Denotes difference compared with the pre-exercise point and between exercise intensities ($P \leq 0.002$) on both trials.



Blood glucose concentration (Table 7.3)

Two-factor repeated measures ANOVA on blood glucose concentration showed a main effect of time ($P = 0.008$), no effect of trial ($P = 0.901$) and no interaction ($P = 0.555$). Blood glucose concentration was lower following low intensity exercise

compared with rest ($P = 0.009$) and the pre-exercise point ($P = 0.001$). Following high intensity cycling, blood glucose was not different from any other time point.

Table 7.3 Blood glucose concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; $n = 8$. * denotes difference from the pre-exercise point ($P < 0.050$). P denotes difference between trials.

	Rest	Pre - exercise	Low Intensity	High Intensity
Water trial	4.62 ± 0.20	4.65 ± 0.33	$4.35 \pm 0.30^*$	4.64 ± 0.35
Glycerol trial	4.53 ± 0.25	4.71 ± 0.32	$4.34 \pm 0.29^*$	4.59 ± 0.25
P	0.530	0.770	0.930	0.800

Blood lactate concentration (Table 7.4)

Two-factor repeated measures ANOVA on blood lactate concentration showed a main time effect ($P = 0.000$), no trial effect ($P = 0.482$) and no interaction ($P = 0.225$). Blood lactate concentration compared with the pre-exercise point increased only during the high intensity stage ($P = 0.000$). Blood lactate between conditions was also similar ($P > 0.050$).

Table 7.4 Blood lactate concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; $n = 8$. * Denotes difference from other time points within trials ($P < 0.050$). P denotes difference between trials.

	Rest	Pre – exercise	Low intensity	High intensity
Water trial	0.7 ± 0.3	0.8 ± 0.1	1.2 ± 0.3	$7.5 \pm 1.6^*$
Glycerol trial	0.6 ± 0.3	1.0 ± 0.2	1.1 ± 0.2	$8.0 \pm 1.7^*$
P	0.290	0.128	0.208	0.282

Serum potassium concentration (Table 7.5)

Two-factor repeated ANOVA on serum potassium concentration showed a main time effect ($P = 0.000$), no trial effect ($P = 0.824$) and no interaction ($P = 0.547$) and that serum potassium concentration increased only at high intensity and was significantly higher than the pre-exercise point ($P = 0.000$) and the low intensity stage ($P = 0.000$).

Table 7.5 Serum potassium concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; n = 8. P denotes difference between trials. * Denotes difference from all other time points within a trial.

	Rest	Pre - exercise	50% VO_2max	100% VO_2max
Water trial	4.7 ± 0.4	4.6 ± 0.3	4.6 ± 0.2	$5.9 \pm 0.7^*$
Glycerol trial	4.5 ± 0.1	4.4 ± 0.2	4.6 ± 0.2	$5.9 \pm 0.7^*$
P	0.217	0.171	0.356	0.680

Serum sodium concentration (Table 7.6)

Two-way repeated measures ANOVA on serum sodium concentration showed a main time effect ($P = 0.000$), no trial effect ($P = 0.324$) and no interaction ($P = 0.553$). Serum sodium concentration increased only at high intensity and it was higher than the pre-exercise point ($P = 0.001$) and sodium concentration at low intensity ($P = 0.002$).

Table 7.6 Serum sodium concentration ($\text{mmol}\cdot\text{l}^{-1}$). Values are means \pm SD; n = 8. P denotes difference between trials. * Denotes difference from all other time points within a trial.

	Rest	Pre – exercise	Low intensity	High intensity
Water trial	142 ± 1	141 ± 1	142 ± 2	$146 \pm 2^*$
Glycerol trial	142 ± 1	141 ± 1	142 ± 1	$146 \pm 2^*$
P	1.000	0.381	0.381	0.697

Exercise results (Table 7.7)

There were no differences between the subjects' previously determined VO_2max ($45.6 \pm 8.6 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and oxygen uptake during the high-intensity exercise stage on the water trial ($P = 0.745$) and on the glycerol trial ($P = 0.691$). A small but significant difference occurred during the low-intensity stage. Oxygen uptake for the same absolute cycling workload was lower on the glycerol trial ($19.9 \pm 4.4 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) compared with the water trial ($21.6 \pm 4.0 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) ($P = 0.005$).

The only other difference between the two trials was time to exhaustion ($P = 0.003$). Participants reached fatigue slightly sooner (23 ± 15 s) on the glycerol trial in 248 ± 62 s, while on the water trial, time to exhaustion was 271 ± 68 s ($P = 0.003$). During exercise, there were no differences between trials in HR or RER. All participants cycled slightly longer on the water trial and only one participant exercised longer on the glycerol trial (Figure 7.6). The individual values of VO_2 obtained during this study are presented in table 7.8.

Table 7.7 Exercise results. Values are means \pm SD; $n = 8$. P denotes difference between trials.

Cycling intensity (W)	Low intensity			High intensity		
	132 \pm 41			319 \pm 73		
	Water trial	Glycerol trial	P	Water trial	Glycerol trial	P
VO_2 ($\text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	21.6 \pm 4.0	19.9 \pm 4.4	0.005	43.1 \pm 7.2	42.2 \pm 7.1	0.265
HR (bpm)	123 \pm 7	121 \pm 6	0.305	179 \pm 5	177 \pm 7	0.055
RER	0.97 \pm 0.10	0.95 \pm 0.06	0.243	1.28 \pm 0.10	1.32 \pm 0.11	0.273
RPE	11 \pm 2	11 \pm 1	0.351	20 \pm 0	20 \pm 0	1.000
Time to exhaustion (s)				271 \pm 68	248 \pm 62	0.003

Figure 7.6 Individual exercise time (s) of all participants on both trials.

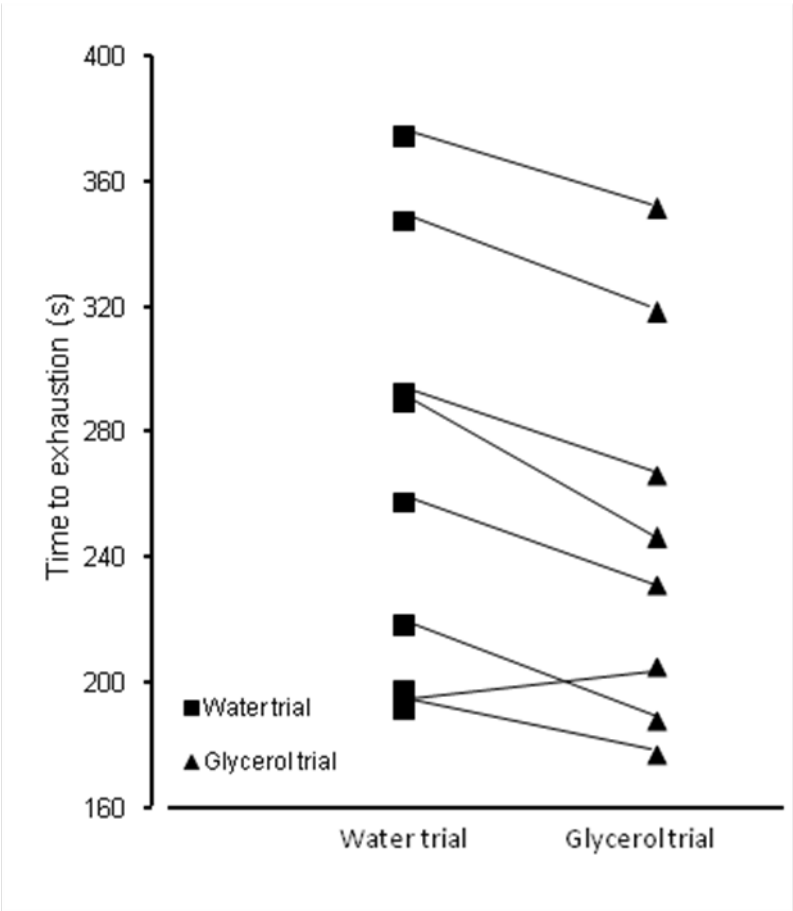


Table 7.8 Individual VO_2max values and VO_2peak obtained on both trials ($\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$).

Participant	VO_2max	Water trial	Glycerol trial	Water trial	Glycerol trial
		VO_2 at low intensity exercise	VO_2 at low intensity exercise	$\text{VO}_{2\text{peak}}$	$\text{VO}_{2\text{peak}}$
	$\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	$\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	$\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	$\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	$\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$
1	37.8	18.5	17.0	37.4	35.9
2	48.2	22.6	20.9	41.7	41.8
3	59.3	30.1	28.1	58.0	54.0
4	54.7	24.0	24.9	46.7	49.0
5	34.8	18.3	16.1	37.5	33.8
6	42.3	20.1	16.6	41.5	42.9
7	40.4	18.6	17.0	36.0	35.3
8	47.9	20.4	18.4	46.3	44.8

Discussion

The aim of this study was to examine any effect of glycerol ingestion and potential altered body water distribution on performance during exercise at 100% VO_2max intensity. Ingestion of glycerol resulted in increased serum osmolality by 16 $\text{mosmol}\cdot\text{kg}^{-1}$. This was accompanied by an increase in blood volume by 4.7%, secondary to a 7.7% increase in plasma volume. The increase in plasma volume was attributed to a shift of water into the vascular space due to increased serum osmolality because of the osmotic properties of glycerol (see chapter 3 of this thesis) leading presumably to a modest state of cell shrinkage (Graf & Haussinger, 1996; Keller et al., 2003). During the exercise, participants reached similar VO_2 levels at the end of exercise on both trials, which were not statistically different from their previously determined VO_2max values, but time to fatigue on the glycerol trial was decreased by a mean of 23 s compared with the water trial. During short exhaustive exercise of 6 – 7 min in duration, the VO_2max is recognised to be an important determinant of performance (Bassett et al., 2000). Exercise duration in the present study was short (~4.1 min on glycerol trial; ~4.5 min on water trial) as exercise intensity was set at a constant high workload, which elicited oxygen utilization levels similar to that of the participants' VO_2max . This intensity was chosen based on the findings of the study described in chapter 6 of this thesis, where during an incremental VO_2max test the only difference between water and glycerol trials was observed only in the very last stage of the test, where participants reached fatigue slightly sooner on the glycerol trial. Despite the short duration of exercise in the present study, it has been shown that in severe-intensity constant work rate exercise, the termination of exercise coincides with the attainment of a VO_2 that is close to the VO_2max attained during incremental exercise (Rossiter et al., 2006; Tordi et al., 2003). In the present study, at high intensity exercise, VO_2 attained was similar to the participants' VO_2max in both trials, but on the glycerol trial, participants reached peak VO_2 and exhaustion sooner, as exercise time was reduced by 8.5% compared with the water trial.

Therefore, it could be deduced that volitional fatigue during high intensity exercise may possibly be dependent on other factors besides VO_2max (Coyle et al., 1986). Mortensen et al. (2005) showed that the limiting factor during cycling against a constant workload (85% VO_2max) was the impaired systemic oxygen delivery due to a decline in stroke volume (and cardiac output) as a result of the restriction in left ventricular filling time and left ventricular end-diastolic volume that accompanies severe tachycardia. Expansion of plasma and blood volume, even when induced acutely (via infusion of a plasma expander) in untrained participants, before starting a single exercise bout, may favour endurance exercise performance due to an increase in blood availability, end-diastolic volume and stroke volume (Coyle et al., 1990; Kanstrup & Ekblom, 1982; Mier et al., 1996). During exercise in the present study, blood and plasma volume fell in both conditions. Water loss to the extravascular space during exercise is attributed to increased interstitial osmolality within working muscles (Hayes et al., 2000). The accumulation of exercise by-products, such as lactate, acts to draw water into the intracellular space, effectively diluting intracellular concentrations to maintain resting conditions (Raja et al., 2006). Damon et al. (2002) demonstrated a significant correlation between lactate, proton production and the resulting intracellular acidification with the increase in intracellular water. The decrease in plasma volume in the present study is in agreement with the changes in plasma water reported in previous studies in literature during cycling (Sjogaard & Saltin, 1982) and dynamic knee extension (Sjogaard et al., 1985). At the end of exercise, blood and plasma volume were higher on the glycerol trial than on the water trial. Therefore, blood availability, and potentially stroke volume, were probably not a limitation for exercise performance on the glycerol trial.

Decreased time to fatigue following plasma volume expansion has also been reported by Coyle et al. (1986) and Kanstrup and Ekblom (1982). In those studies, plasma volume expansion was elicited via infusion of 700 ml dextran. Despite the positive effect of improved stroke volume and cardiac output, the resulting haemodilution of 8 – 11% drastically reduced the blood's oxygen carrying capacity

and delivery to the exercising muscles. It appears that plasma volume expansion greater than 650 ml has a detrimental effect on aerobic exercise performance in untrained participants (Coyle et al., 1990; Kanstrup & Ekblom, 1982, 1984). In the present study, on the glycerol trial, haemoglobin concentration was slightly (statistically non-significantly) decreased by 5% and the increase in plasma volume was 7.7%. According to the literature, a moderate plasma volume expansion (250 – 500 ml) which results in a moderate haemodilution of 4 – 5% appears to have a positive effect on aerobic exercise performance, as the improvement in cardiac output offsets the mild haemodilution, resulting in improved performance (Coyle et al., 1990; Krip et al., 1997; Luetkemeier & Thomas, 1994). According to the literature, these moderate alterations of haemoglobin and plasma volume should have a positive effect on exercise performance and time to fatigue. An explanation for this discrepancy between the literature and the results of the present study may lie in the methods used to elicit plasma volume expansion. In other studies, participants were well hydrated and a plasma volume expander was infused to further increase plasma volume. In the present study, participants ingested a glycerol solution that possibly resulted in alterations of body water distribution, and the increase of plasma volume may have potentially been at the expense of intracellular water and muscle hydration (Hamilton et al., 1993).

It is likely that the increases of extracellular osmolality on the glycerol trial induced a modest state of cell shrinkage (Blinks, 1965; Graf & Haussinger, 1996; Hamilton et al., 1993; Keller et al., 2003). A decrease in cell hydration has been shown to elicit increases in blood lactate (Antolic, 2007; Lang et al., 1989). In the present study, blood lactate concentration were similar between the two conditions, but peak blood lactate value on the glycerol trial occurred in less time; this could suggest an accelerated lactate production or efflux, as exercise time was slightly shorter on the glycerol trial. Because these blood samples was taken at the end point of high intensity exercise it is not, however, clear whether samples taken at the same chronological time points during this stage of exercise would have yielded lower lactate levels on the glycerol trial compared with the water trial.

Furthermore, blood volume on the glycerol trial was higher than that on the water trial, which suggests a higher net lactate production on the glycerol trial (Kanstrup & Ekblom, 1982). Lactate production is accompanied by hydrogen ion accumulation which is a fatiguing factor, and this might have contributed to the fact that exhaustion occurred sooner on the glycerol trial.

Fatigue may also result from a substantial loss of K^+ from the active muscles; its accumulation in the extracellular space and the associated decline in the K^+ concentration difference across the sarcolemma might have promoted exhaustion, as muscle excitability and contraction depends on the K^+ concentration gradient across the muscle surface membrane (Sejersted & Sjogaard, 2000; Sjogaard et al., 1985). In vitro, it has been shown that membrane depolarization caused by accumulation of extracellular potassium leads to the development of muscular fatigue (Cairns et al., 1995). Even though potassium is lost from the exercising muscles at all intensities, at low intensities, there is sufficient re-uptake of potassium to maintain intracellular concentration, but at high intensity, there is a mismatch between K^+ loss and re-uptake and, as a result, venous potassium has been shown to rise throughout the exercise period to a maximum value at exhaustion (Vollestad et al., 1994). In the present study, serum potassium concentration was measured before exercise, at the end of the low-intensity phase and at the end of exercise. Serum potassium rose in both trials only during high-intensity exercise, showing increased extracellular accumulation of potassium. At exhaustion, serum potassium concentration was similar on both trials, but exercise duration was slightly shorter (23 s) on the glycerol trial. This could, perhaps, suggest a larger release of potassium from the muscle into the extracellular space on the glycerol trial, which possibly affected endurance. This suggestion is in agreement with in vivo evidence that during exhaustive leg exercise, a faster accumulation of interstitial K^+ , which was induced by prior arm exercise, was associated with a reduced time to fatigue (Bangsbo et al., 1996; Nordsborg et al., 2003). However, it is unclear in the present study, whether serum potassium levels were really different between trials at the same chronological time points.

Some other factors and assumptions can be taken into consideration, bearing in mind that these derive from in vitro studies. Caputo (1968) showed that exposure of muscle cells to a hypertonic medium containing glycerol resulted in cell shrinkage that was accompanied by a reduction in twitch force, which was reversed upon return to the normal isotonic medium. In accordance with these findings, Suarez-Kurtz and Sorenson (1977) reported that hyper-osmotic glycerol solutions have an inhibitory effect on muscle membrane Ca^{2+} activation, which may negatively affect muscle contractility. In a different study, Devin et al. (1996) reported that hyperosmolality resulted in decreased mitochondria matrix volume and a reduction in ATP synthesis due to decreased oxidative phosphorylation efficiency. More recently, while investigating the effects of extracellular osmolality on muscles, Antolic et al. (2007) reported that in hypertonic conditions, muscle water decreased and this was accompanied by decreased muscle ATP and phosphocreatine contents, increased creatine, inorganic phosphate and lactate contents. Muscle contractility could have been affected by the decrease in hydration. Furthermore, lactate with the accompanying hydrogen ions, along with increased inorganic phosphate, have all been linked to fatigue (Allen & Westerblad, 2001). None of the above can be confirmed by the data collected in the present study nor can the exact mechanism that led to fatigue on the glycerol trial be confirmed.

Conclusion

Glycerol ingestion and the subsequent blood and plasma volume increase did not increase time to fatigue during high-intensity exercise at 100% $\text{VO}_{2\text{max}}$. On the contrary, time to exhaustion decreased slightly on the glycerol trial. This may have been due to a negative effect of high extracellular osmolality on muscle metabolism and function. As most of the evidence regarding cell hydration and how that affects cell function derives from in vitro studies, the magnitude that extracellular tonicity may affect cell volume, metabolism and function in vivo remains elusive. More research is required to shed light on the cell volume theory in a whole body situation during exercise.

Chapter 8

General Discussion

Body water distribution – effects of glycerol ingestion

The purpose of this thesis was to indirectly assess in vivo the cell volume theory, proposed by Haussinger in the 1990s, in a whole body situation in an exercising environment. In vitro evidence shows that hypo-osmotic cell swelling counteracts proteolysis and glycogen breakdown in the liver, whereas hyper-osmotic cell shrinkage promotes protein breakdown, glycolysis and glycogenolysis (Haussinger et al., 1990, 1991, 1994). In vitro exposure of muscle fibres to a hypertonic environment has been shown to inhibit contractility when the fibres were electrically stimulated (Rapoport et al., 1982).

Exposure of muscle fibres to hypertonic solutions in vitro is known to lead to cell dehydration and shrinkage (Blinks, 1965; Bozler, 1965). In vivo high concentration glycerol solutions have been reported to be a potent dehydrating agent (Frank et al., 1981), and high elevation of extracellular osmolality following glycerol feeding may well result in cell dehydration and tissue shrinkage (Gleeson et al., 1986; Maughan, 1998; Nelson & Robergs, 2007) as the distribution of fluid between intracellular and extracellular compartments is determined mainly by the osmotic gradient across the cell membrane (Guyton & Hall, 1996; Maughan, 1998).

The study reported in Chapter 3 of this thesis was designed to indicate the potential changes in body water distribution following ingestion of glycerol in a small-volume, highly-hyper-osmotic solution (400 ml solution, 1 g·kg⁻¹ glycerol), and to establish the time course of this effect. Blood volume increased following glycerol ingestion, secondary to an increase in plasma volume, suggesting an alteration in body water distribution, as no real change had occurred to the total body water volume. From the data obtained it was not possible to distinguish whether the increased amount of water in the vascular space originated from the interstitial or intracellular space, or from both of these pools. Data from in vitro studies suggest that water movement due to high extracellular osmolality does indeed reduce intracellular water volume (Hamilton et al., 1993; Lindinger et al., 2011); and in vivo induced high serum osmolality, which was presumed to promote

cell volume shrinkage, has been reported to lead to alterations in the regulation of intracellular metabolic pathways (Berneis et al., 1999; Keller et al., 2003). Based on the presumption that glycerol ingestion likely resulted in a modest reduction of muscle hydration, glycerol ingestion was then used in the other studies reported in this thesis as a means of altering body water distribution in order to investigate potential effects of increased extracellular osmolality and a possible reduced muscle cell hydration on acute strength exercise (chapter 4 and chapter 5), over an 8-week strength training programme (chapter 5), and the effects of increased blood volume availability combined with potential decreased muscle hydration on oxygen uptake and time to fatigue (chapter 6 and chapter 7).

Effects of altered body water distribution on strength

Previous studies on isometric strength and dehydration have reached conflicting results. Torranin et al. (1979) showed that isometric endurance decreased following 4% dehydration by sauna exposure. Viitasalo et al. (1987) reported decreased maximal isometric leg strength (7.8%) following exposure to sauna. Schoffstall et al. (2001) reported that passive dehydration resulting in 1.5% body mass dehydration decreased maximum bench press performance. On the other hand, Greiwe et al. (1998) reported no change in isometric knee extension peak torque or in time to fatigue following 3.8% body mass dehydration (sauna exposure). Montain et al. (1998) showed that after a 4% loss in body mass following active dehydration, maximum voluntary contraction (MVC) during single-leg knee extensions was not affected, but endurance (time to fatigue) was reduced by 15%.

The difference between the studies reported in chapters 4 and 5 and the studies referenced above is that in the present study, there was no reduction in total body water, only an alteration of body water distribution and possibly a reduction in muscle cell hydration due to the distribution and osmotic properties of ingested glycerol. In the study reported in chapter 4, participants were asked to perform isometric quadricep activations at 90% MVC, while participants in the study reported in chapter 5 were asked to execute 4 sets of 10 repetitions of handgrip

exercises at 80% MVC. The results showed that glycerol ingestion and the subsequent water shifts, did not have any acute effect on strength. This is in agreement with some of the previous studies on dehydration and strength in the literature (Bigard et al., 2001; Greenleaf et al., 1967; Greiwe et al., 1998; Montain et al., 1998).

The important finding in the study reported in chapter 4 was the difference in blood lactate concentration, which was higher on the glycerol trial than on the water trial during the recovery period, suggesting possible altered muscle metabolism. This elevated blood lactate in the recovery period could be attributed to a regulatory volume increase mechanism. In vitro evidence showed that exposure of cells to a hypertonic medium results in increased glycogenolysis, which leads to increased lactate production (Antolic et al., 2007; Lang et al., 1989). Lactate accumulates in the muscle cell, thereby intracellular osmolality increases, water is drawn into the cell and cell volume increases (Usher-Smith et al., 2009). This mechanism has been suggested to occur in an effort for the muscle cell to maintain its volume, although the specific mechanism(s) that result in the increased activity of the glycolytic machinery and lactate accumulation are unclear (Antolic et al., 2007). In the present study, if the increase in blood lactate on the glycerol trial was an indication of reduced cell hydration and volume, and if muscle dehydration persisted in the recovery period, it could potentially have a long-term effect on strength exercise and training, as muscle dehydration in vitro has been shown to counteract anabolic processes such as protein synthesis (Haussinger et al., 1994). Measures involving humans and animals (Ritz et al., 2003) have shown that cell volume shrinkage signals a temporary halt in protein formation. Judelson et al. (2008) showed that performing strength exercise in a dehydrated state (5% body mass) attenuates the exercise response, as catabolic hormones cortisol and norepinephrine were higher than in a control trial; furthermore, the response of the anabolic hormones testosterone and insulin were attenuated, producing a less beneficial post-exercise milieu.

The study reported in chapter 5 was designed to investigate long-term effects of exercising and recovering under high extracellular osmolality and a potential

reduced muscle cell hydration following ingesting of a glycerol solution before and immediately after exercise (total glycerol solution $1\text{g}\cdot\text{kg}^{-1}$, $6\text{ ml}\cdot\text{kg}^{-1}$ body mass). Two groups of participants undertook the same handgrip strength training regimen for 8 weeks. One group ingested water and the other group ingested the glycerol solution immediately before and after each training session. The results showed that maximum handgrip strength increased in both groups to the same extent. Mean strength in the water group increased by 15% and in the glycerol group by 8%. The small number of participants (7 in each group) and the large standard deviation observed between participants might have prevented the results between groups from reaching a statistical significant difference.

Future research on muscle hydration and strength should possibly incorporate a more strenuous strength exercise regime using multiple exercises in a single workout and training a greater muscle mass, or the training could be extended for at least 12 weeks. Measurements such as hormonal response and more invasive methods in measuring extracellular and intracellular cell hydration from muscle biopsies and protein metabolism could be used to shed light onto the role of muscle cell hydration in a whole body situation.

Effects of altered body water distribution on maximum oxygen uptake and time to fatigue

Endurance trained individuals exhibit higher blood volume and total haemoglobin levels than sedentary individuals (Kjellberg et al., 1949). A major adaptation to endurance training is an increase in stroke volume resulting from an increase in circulating blood (Clausen, 1977; Coyle et al., 1986), because of the Frank-Starling mechanism (Coyle et al., 1990; Ekblom, 2000; Fortney et al., 1981; Kanstrup & Ekblom, 1982), as increased ventricular filling pressure leads to increased output (stroke volume). Plasma volume expansion independently of increases in haemoglobin and haematocrit leading to expansion of blood volume has been shown to increase VO_2max , especially in untrained participants who do

not have an elevated blood volume as a result of endurance training (Coyle et al., 1986, 1990; Krip et al., 1997).

In the studies reported in chapter 6 and chapter 7 of the present thesis, participants ingested a glycerol solution ($1 \text{ g} \cdot \text{kg}^{-1}$ body mass, $6 \text{ ml} \cdot \text{kg}^{-1}$ body mass), which resulted in a 5% increase in blood volume secondary to a 7.7% increase in plasma volume and a slight non-significant 5% decrease in haemoglobin concentration. A moderate plasma volume expansion (250 – 500 ml) that results in a moderate haemodilution of 4 – 5% appears to have a positive effect on aerobic exercise performance, as the improvement in cardiac output offsets the mild haemodilution, resulting in improved performance (Coyle et al., 1990; Krip et al., 1997; Luetkemeier & Thomas, 1994).

In the study reported in chapter 6 of this thesis, participants performed an incremental VO_2max test on a cycle ergometer under control and blood volume expanded conditions. The results showed that VO_2max did not improve on the glycerol trial and that time to fatigue decreased. In the study reported in chapter 7 participants cycled to fatigue against a constant workload corresponding to their VO_2max intensity under control and blood volume expansion due to glycerol ingestion. The results showed that participants matched their VO_2max in both conditions, but time to fatigue was decreased on the glycerol trial. This is contrary to the findings of Berger et al. (2006), Coyle et al. (1990) and Luetkemeier and Thomas (1994) who found improvements in aerobic performance promoted by hypervolaemic-induced increase in stroke volume.

The current belief is that maximum cardiac output is the principal limiting factor for VO_2max (Basset et al., 2000; Ekblom, 2000; Gonzalez-Alonso & Calbet, 2003) as a result of the impaired systemic oxygen delivery due to a decline in stroke volume (and cardiac output) caused by the restriction in left ventricular filling time and left ventricular end-diastolic volume that accompanies severe tachycardia. In the present studies, blood volume at the end of exercise was higher on the glycerol trial than on the water trial, so blood volume availability was presumably not a limiting factor.

The slightly faster rate of fatigue on the glycerol trials in the studies reported in chapters 6 & 7 may have occurred due to peripheral reasons. Fatigue is traditionally associated with high intracellular accumulation of H^+ , which accompanies lactate production. In the studies reported in chapter 6 and chapter 7, blood lactate concentration was similar between the two experimental conditions, but exercise on the glycerol trial ended slightly sooner, perhaps suggesting, an accelerated lactate production or efflux. It is possible that lactate production on the glycerol trial was greater. In vitro, osmotic cell shrinkage has been shown to increase lactate release from the muscle (Antolic et al., 2007; Lang et al., 1989), and in the studies described in chapters 6 & 7 the alteration of body water distribution on the glycerol trials had likely resulted in a modest decrease in muscle water.

Fatigue may also result from increases in extracellular K^+ concentration, which alters muscle excitability and contraction (Sejersted & Sjogaard, 2000; Sjogaard et al., 1985). During high intensity exercise, there is a mismatch between myocyte K^+ loss and re-uptake and, as a result, venous potassium concentrations have been shown to rise throughout the exercise period, to a maximum value at exhaustion (Vollestad et al., 1994). In the study reported in chapter 7, serum potassium concentration was higher at the end of exercise compared with rest and 50% VO_{2max} intensity. Comparisons between the two experimental conditions, showed that serum potassium concentration was similar, but exercise time on the glycerol trial was, again, slightly shorter (23 s). Although this time difference is small, it is possible that a larger release of potassium from the muscle into the extracellular space occurred on the glycerol trial. In vivo evidence has shown that during exhaustive leg exercise, a faster rate of accumulation of interstitial K^+ (which was induced by prior arm exercise) is associated with a reduced time to fatigue (Bangsbo et al., 1996; Nordsborg et al., 2003). Therefore the potential of a fatigue-inducing higher K^+ release from the muscle on the glycerol trial cannot be excluded.

From the data collected in the present thesis, no conclusive remarks can be made concerning what affected exercise performance and accelerated the development of fatigue following glycerol ingestion. More in-depth research is required to investigate how increased extracellular osmolality and possible altered muscle hydration status affect muscle function and metabolism in vivo. Perhaps more invasive methods would be able to reveal how the intramuscular environment is affected by hyper-osmolality and cell volume shrinkage in a whole body situation.

Conclusions

Ingestion of a small-volume, highly-hypertonic glycerol solution results in altered body water distribution, as indicated by an increase in plasma and blood volume. High serum osmolality and the potential altered body water distribution appear to have no acute effect on force production during isometric exercise at high intensity. Any potential altered body water distribution during and immediately after exercise following glycerol ingestion does not seem to affect the training outcome of an 8-week handgrip training programme, but further investigation is required.

High serum osmolality and the potential altered body water distribution leads to a slight but significant decrease in time to fatigue during cycling at high intensity. Further research is required to investigate the mechanism that accelerates this fatigue development.

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Appendix A

Detailed description of various haematological assays

Measurement of haemoglobin concentration

Haemoglobin concentration was determined with the cyanmethaemoglobin method, where haemoglobin is converted to cyanmethaemoglobin and measured photometrically.

Blood samples collected during the experimental studies were dispensed in K₂EDTA containing tubes. The tubes were then placed on racks until the end of the experimental session (<2.5 hours) and then stored temporarily in the refrigerator (<4° C) until analysis time. All blood samples were analyzed for haemoglobin on the same day of collection.

Prior to blood analysis the tubes were placed on a roller mixer so that the blood was uniformly mixed.

Sample analysis

In duplicate, 10 µl of blood sample was added to 2.5 ml of Drabkins reagent in small glass tubes and mixed thoroughly. Special care was taken when pipetting and dispensing the blood due to the small quantity of blood needed for this assay. A tissue was used to wipe off any excess blood from the outside surface of the pipette tip. Adequate pipetting skills training and practice took place prior to blood and data collection for this thesis.

Following mixing of blood with the reagent in small glass tubes, the samples were left to incubate for 30 min at room temperature, away from direct sunlight.

Haemoglobin standards were previously prepared in the laboratory from blood and Drabkins reagent and were calibrated against a commercial standard. Always the haemoglobin concentration of the standards was 180 g·l⁻¹ or higher.

Following incubation, the standards and samples absorbance was read by the spectrophotometer at a wavelength of 540 nm. In order to do so the standards and the samples were dispensed in a plastic cuvette, which was then placed in the appropriate slot in the spectrophotometer. The same cuvette was used for all

standards and samples. Special care was taken to remove all fluid from the cuvette (by giving it a strong shake) before dispensing the next sample in it.

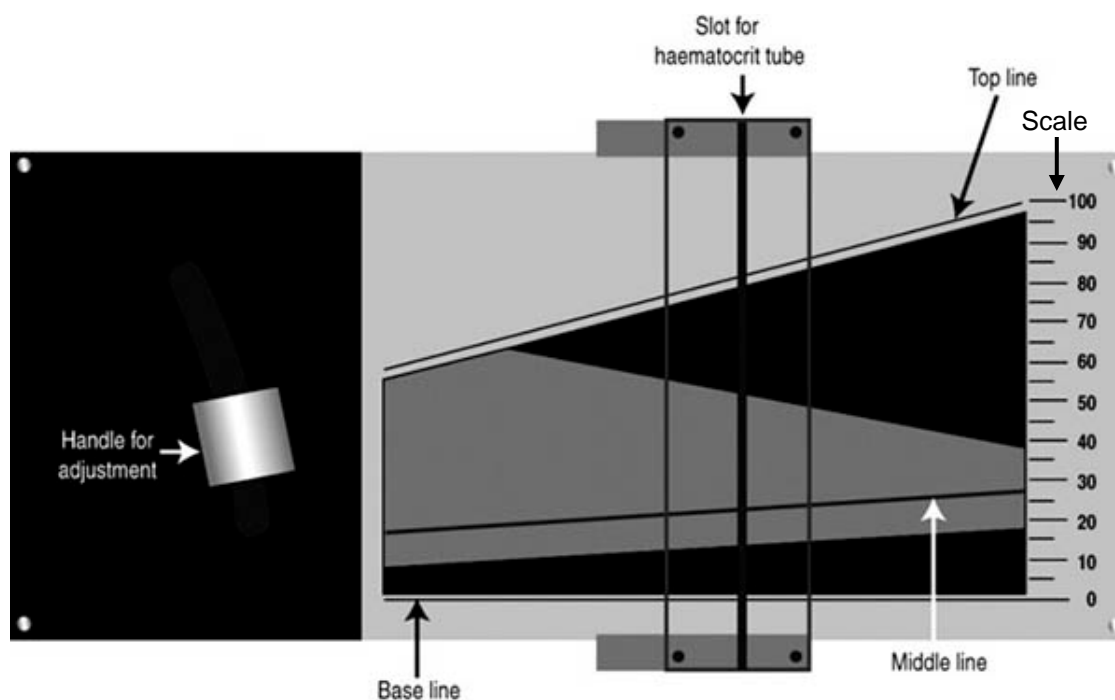
Measurement of red blood cells volume (Haematocrit)

Haematocrit is the volume occupied by the red blood cells compared with the overall volume of a column of whole blood, expressed as a percentage of the whole blood volume (e.g. 45%).

Plain capillary tubes were dipped in tubes containing EDTA treated blood, and blood was drawn into plain capillary tubes by simple capillary attraction. Care was taken that there were no air bubbles formed in the capillary tube, which was filled to approximately 2/3 of its length. The capillary end not dipped in blood was sealed by inserting and twisting that end into a block of clay type sealant. Then, the outside of the capillary tube was wiped cleaned. Each sample was measured in triplicate. All capillary tubes were placed in the rotor of a micro-haematocrit centrifuge. Each capillary tube lay in one of the channels in the rotor with the sealed end resting against the rubber rim of the centrifuge. When there were not enough capillary tubes to fill all channels of the rotor, care was taken than the tubes were placed in such a way to provide equal balance around the rotor. Then, the centrifuge lid was closed tightly and the samples spun for 5 min.

Following centrifugation, each spun tube was placed in the slot of the haematocrit reader. The base line of the reader was aligned with the bottom of the red blood cells column (upper end of the sealant plug). By using the handle, the middle line was adjusted to intersect with the top of the red blood cell column. Haematocrit was read at the point where the middle line cut the scale. Caution was taken to look directly down at the reader when lining up the capillary tube with the lines on the reader as this minimizes readings errors.

Figure A.1. Haematocrit reader



Measurement of blood glucose concentration

Blood glucose concentration was determined by the GOD-PAP method (Randox). Upon collection of the blood sample 100 μ l of whole blood was deproteinised by being added into an eppendorf tube containing 1000 μ l of ice cold 0.3 M perchloric acid, in duplicate. As the blood tends to stick to the inside wall of the plastic pipette tip, the pipette was rinsed several times by depressing the pipette button half-way and releasing it several times before finally dispensing the mixture into the eppendorf tube. Then, the eppendorf tube was given a good shake for better mixing of the blood with the perchloric acid. Following collection of all blood samples, the eppendorf tubes were spun in an eppendorf centrifuge for 1 min and were then stored in a refrigerator ($<4^{\circ}\text{C}$). The supernatant collected was used for blood glucose determination on the same day.

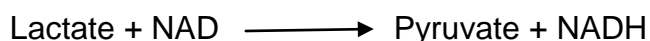
Sample analysis

In duplicate, 200 µl of supernatant was added to 2000 µl of Randox reagent in small glass tubes. For the blank standard, 200 µl of distilled water was added to the reagent. The top standard used was a 5.55 mmol·l⁻¹ glucose stock solution, previously prepared in the laboratory, which was compared against a commercially available standard. The samples were left to incubate for 30 min at room temperature, away from direct sunlight. Following incubation, the standards and samples absorbance was read by the spectrophotometer at a wavelength of 500 nm, using a single cuvette as already described above for the haemoglobin assay.

Measurement of blood lactate concentration

The supernatant collected from blood samples and perchloric acid (as described above for blood glucose determination) was frozen (-20° C) and was later used for determination of blood lactate concentration.

The method used was based upon that of Maughan (1982) which is dependent upon the release of NADH during the follow reaction



For the lactate assay, hydrazine buffer (pH 9.4) and a reaction mixture were needed. The reaction mixture was prepared immediately prior to use, containing 2.0 mg NAD and 10.0 µl LDH per 1 ml hydrazine buffer. This made 1 ml of reaction mixture. 200 µl of hydrazine buffer was required per fluorimeter tube for the assay. Hydrazine buffer was made by 6.5g hydrazine sulphate and 24.26 ml hydrazine hydrate, diluted in distilled water to make 500 ml hydrazine buffer. Enough reaction mixture was prepared for a batch of samples in duplicate and for >17 more tubes of blanks and standards: 4 blank tubes, 4 top standard tubes and various in between standards (at least three) in triplicate. Furthermore, extra reaction mixture enough for 5 tubes was prepared to allow for some loss in pipetting. One blank and one top standard tube were used to set the range on the

fluorimeter. Once the tube was read it was not used again as the fluorescence changes. The standard curve was always found to be linear.

The standards were made from 1.0 M sodium L-Lactate stock solution, representing concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 and 20.0 mmol·l⁻¹.

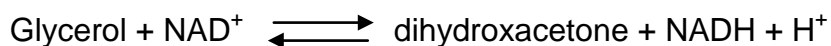
Different concentration standards were used for each batch of samples according to the expected range of results.

Sample analysis

The samples and standards were removed from the freezer and were allowed to thaw at room temperature for at least one hour. Then, 20 µl of either standard or supernatant was dispensed into a pyrex fluorimeter tube and 200 µl of reaction mixture was added. The supernatant was thoroughly mixed with the reaction mixture, and the tubes were allowed to incubate for 30 min. The tubes were covered with a sheet of paper to prevent contamination. Following incubation, 1.0 ml of distilled water was added to the tubes and then fluorescence of the blanks, standards and samples were read on the fluorimeter.

Measurement of serum glycerol concentration

Determination of serum glycerol concentration was done based on the method by Boobis and Maughan (1983), which is dependent upon the release of NADH during the follow reaction:



For the glycerol assay a reaction mixture made of hydrazine buffer, NAD 20 µl (1 mmol·l⁻¹ final concentration) and GDH 5 µl (2.5 U·l⁻¹ final concentration) were needed. All details regarding these reagents can be found in the original paper by Boobis and Maughan (1983). Caution must be taken so that the buffer has pH 9.9 for the above chemical reaction to proceed to the right to completion in order to get correct readings of serum glycerol.

Sample analysis

Following collection of serum samples, 20 μl of serum was added to 200 μl of ice cold 0.3 M perchloric acid in eppendorf tubes. Then the eppendorf tubes were spun for 1 min in an eppendorf centrifuge, and the supernatant collected was used for determination of glycerol. The samples were frozen (-20°C) until analyzed. Glycerol stock solution ($2\text{ mmol}\cdot\text{l}^{-1}$) was also stored at -20°C in small eppendorf tubes.

On analysis day, the samples and standards were removed from the freezer and were allowed to thaw at room temperature for at least one hour. Different working standards were prepared fresh as needed. Low glycerol concentration standards (expected values below $0.075\text{ mmol}\cdot\text{l}^{-1}$) were analyzed separately from samples taken following glycerol ingestion where glycerol concentration was expected to be very high. Low glycerol standards were prepared as described in the method by Boobis and Maughan (1983). For samples where high glycerol concentration was expected, glycerol standards of 0, 0.5, 1.0, and $1.5\text{ mmol}\cdot\text{l}^{-1}$ glycerol concentration were made, and following fluorescence reading glycerol concentration for the standards and samples was corrected for dilution (multiplied by 11).

In duplicate (triplicate for the standards), 50 μl of blank, standard and sample was dispensed in pyrex fluorimeter tubes. Then 200 μl of reaction mixture was added and mixed thoroughly with the sample. Enough reaction mixture was prepared for a batch of samples in duplicate, for blanks, standards and for 5 tubes extra to allow for some loss in pipetting. The tubes were left to incubate for 90 min in room temperature and they were covered with a sheet of paper to prevent contamination. Following incubation, 1 ml of $20\text{ mmol}\cdot\text{l}^{-1}$ sodium carbonate buffer was added to each tube. Then, the range on the fluorimeter was set with the use of extra blank and top standard containing tubes and fluorescence was measured for the standards and samples. Glycerol concentration of the samples was calculated from the linear standard curve.

Appendix B

Pilot study on potential effects of glycerol ingestion on blood pressure

Introduction

Prior to the studies on glycerol ingestion and cycling exercise, the ethical advisory committee of Loughborough University raised the question whether glycerol ingestion and the resulting increase in plasma volume would increase blood pressure, and whether such an increase would pose a safety issue during cycling. In order to answer this question four volunteers (3 male, 1 female) cycled on two occasions following ingestion of either lemon- flavoured water ($6 \text{ ml} \cdot \text{kg}^{-1}$ body mass) or a lemon-flavoured glycerol solution ($1 \text{ g} \cdot \text{kg}^{-1}$ body mass glycerol; $6 \text{ ml} \cdot \text{kg}^{-1}$ body mass total solution volume).

After the participants arrived at the laboratory they were seated comfortably for a period of 20 min, and then the first blood pressure measurement was taken using the Omron MS-I automatic blood pressure monitor (Omron Healthcare, Inc., USA). Immediately after, the subjects ingested either lemon flavoured water or the lemon flavoured glycerol solution and remained seated for a further 25-min period before moving onto the cycle ergometer. Exercise commenced 30 min after ingestion of water or the glycerol solution, as at this time point plasma volume expansion reaches its peak value following glycerol ingestion. Exercise consisted of 5 min cycling on an electrically braked cycle ergometer (Gould, Bilthoven, The Netherlands) at a constant intensity equal to 50% of the maximum workload achieved during a previously performed VO_2max test. The cycle ergometer seat was adjusted according to the height of each participant. The participants were allowed to pedal at a cadence of their choice, but higher than 60 rpm. Two blood pressure measurements were taken when the participants were on the bike; one resting measurement before exercise and another measurement during the last minute of exercise whilst the participants were still pedalling. In order to take blood pressure measurements the participants would release the left arm from the bicycle handle bars and tried to maintain minimum movement of the upper body. Attempts to measure blood pressure at higher cycling intensities often ended in

failure as there was great body movement by the participants and valid blood pressure readings could not be obtained.

Results

Blood pressure measurements showed that blood pressure was not affected by glycerol ingestion and the subsequent increase in plasma volume. Systolic blood pressure during submaximal cycling was (mean \pm SD) 161 \pm 16 mmHg on the water trial and 156 \pm 19 mmHg on the glycerol trial. Diastolic blood pressure was 80 \pm 6 mmHg on the water trial and 72 \pm 6 mmHg on the glycerol trial. Table 1 below shows systolic blood pressure of each participant.

Table 1. Systolic blood pressure (mmHg).

Participant	Water trial			Glycerol trial		
	Rest	Rest on bike	Exercise	Rest	Rest on bike	Exercise
1	120	117	168	120	120	146
2	125	125	153	119	120	156
3	120	120	142	99	94	139
4	140	140	179	127	133	183
Mean	126	126	161	116	117	156
\pm SD	\pm 9	\pm 10	\pm 16	\pm 12	\pm 16	\pm 19

Conclusion

From the above results it was obvious that compared to a control trial, glycerol ingestion does not increase blood pressure during rest, nor at a submaximal cycling intensity.

From data taken previously in the laboratory, plasma volume increases by ~7.7% and blood volume increases by ~4.5% following glycerol ingestion. These

changes in plasma and blood volume are moderate in comparison with other studies (Table 2) where plasma volume expanders were used. Blood pressure was monitored in the studies described in table 2 and there was no difference between control and plasma volume expansion conditions.

Table 2. Studies on plasma and blood volume expansion which measured blood pressure. PV – plasma volume, BV – blood volume, BP – blood pressure.

Authors	PV and BV expansion	Type of exercise used	Blood pressure
Hopper et al., 1988	PV↑ 20% via infusion of 6% dextran solution	Cycling at 50% and 60% VO ₂ max	BP measured sphygmomanometrically. No difference in BP between conditions.
Kanstrup and Ekblom, 1982	BV↑ 12.6% via infusion of 500 – 1000 ml of 6% dextran solution	Cycling at 100, 150, 200W and maximal intensity running exercise to exhaustion	BP measured via an arterial catheter. No difference in BP between control and BV expansion conditions.

Other studies also studied acute effects of plasma and blood volume expansion on aerobic exercise but without monitoring blood pressure (Table 3). In these studies there were no reports of any adverse health effects following plasma and blood volume expansion.

Table 3. Studies on plasma and blood volume expansion that did not monitor blood pressure.

Authors	PV and BV expansion	Type of exercise used
Zavorsky et al., 2003	BV↑ 8.3% via infusion of 500 ml Pentaspan	6.5 min cycling at ~92% VO ₂ max Running at 75%
Coyle et al., 1990	PV↑ 11% via infusion of 6% dextran solution	VO ₂ max, 95-100% VO ₂ max, and 110% VO ₂ max

Based on the data collected during this pilot study and from other studies in the literature, it was safe to assume that glycerol ingestion and the subsequent blood volume expansion did not pose any health risks regarding blood pressure.

Furthermore, it was made certain that all participants in the cycling studies would be recreationally active, accustomed to cycling and high intensity exercise, as they would be asked to reach volitional exhaustion.

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